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.

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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].

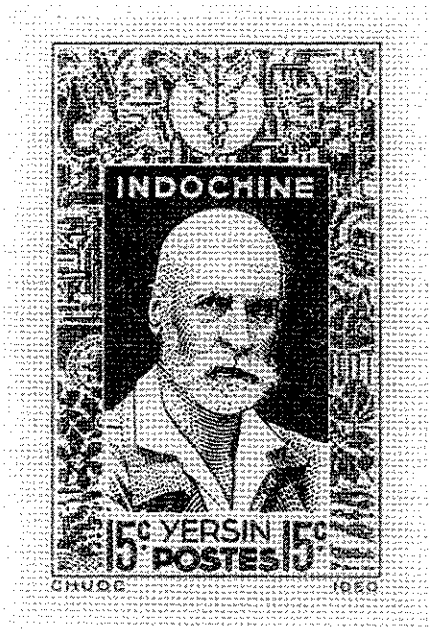
1940s - 1960s: Secluded existence

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].

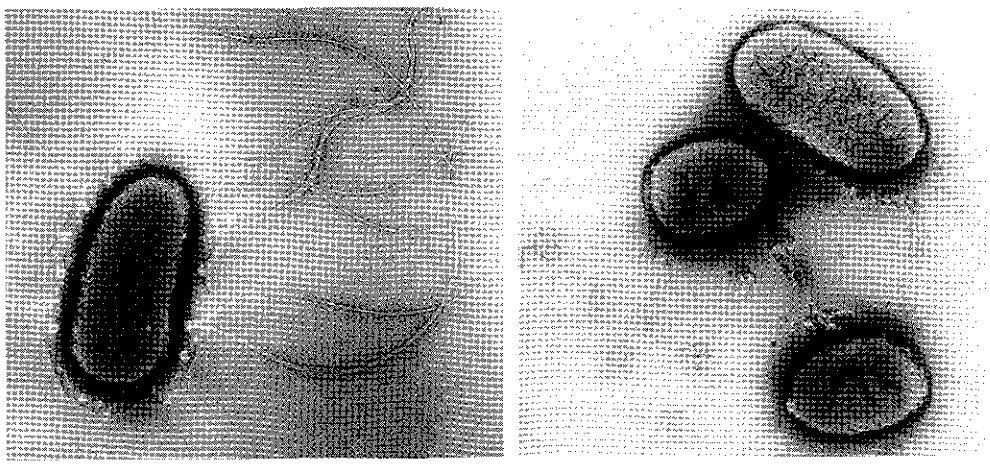


Figure 1.1 *Yersinia enterocolitica.* On the leftside: cells grown at 5°C; rightside: cells grown at 37°C

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).

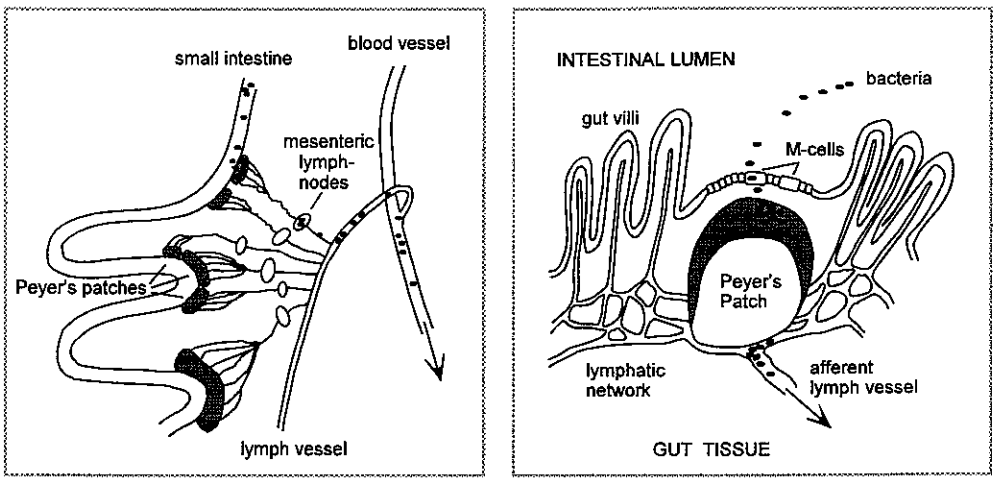


Figure 1.2 Entrance and spread of *Yersinia enterocolitica* in the human host

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].

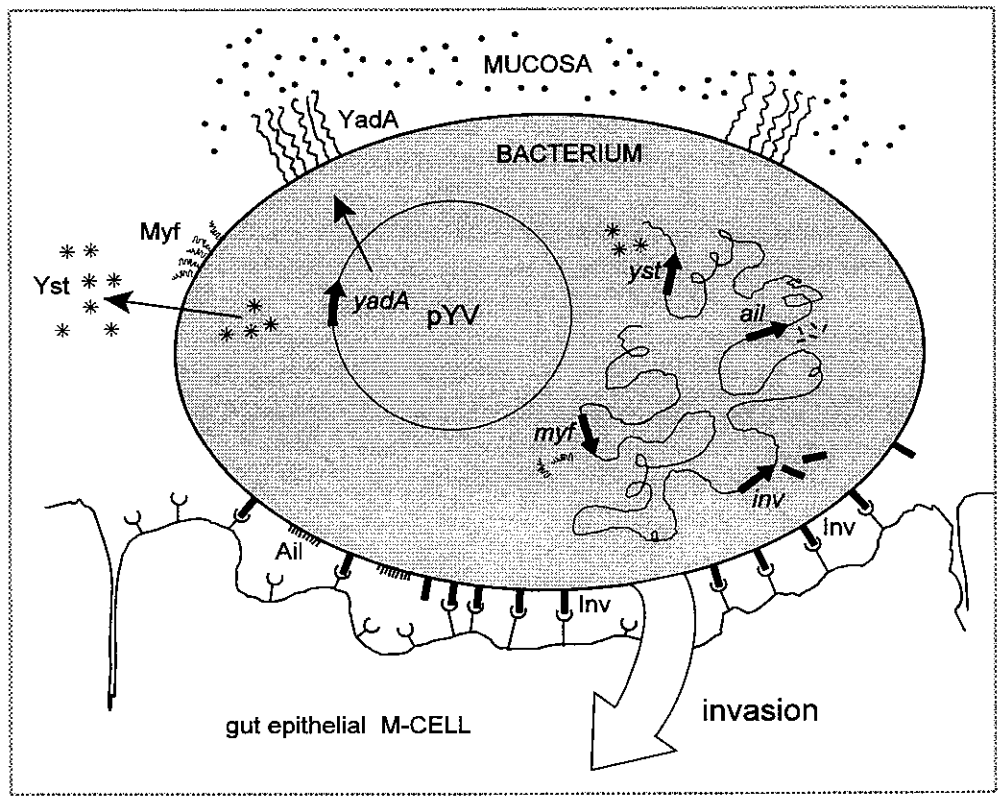


Figure 1.3 Yersinia enterocolitica in the intestine:
genes and gene-products which are involved in the invasion of the host epithelial tissues

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.

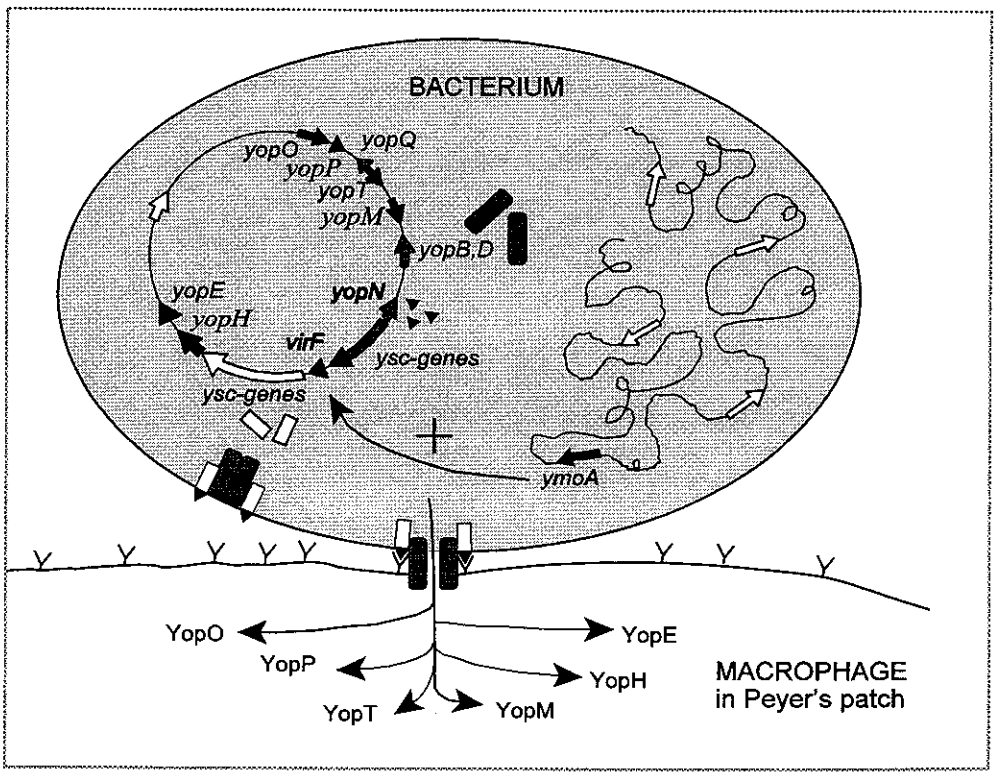


Figure 1.4 *Yersinia enterocolitica* in the Peyer's patch: genes and gene-products involved in the process of anti-phagocytosis.

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].

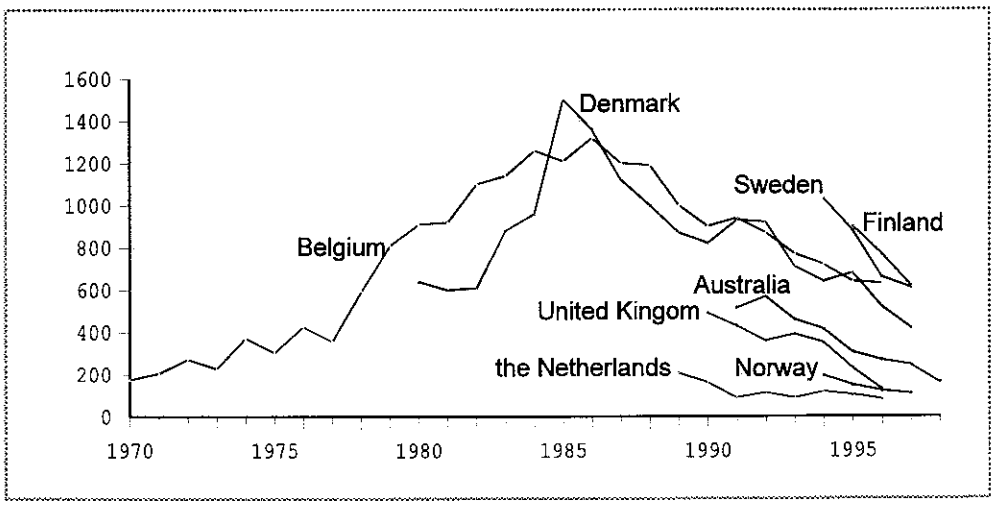


Figure 1.5 Trends in the incidence of yersiniosis:
amounts of annually reported cases in various countries

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].

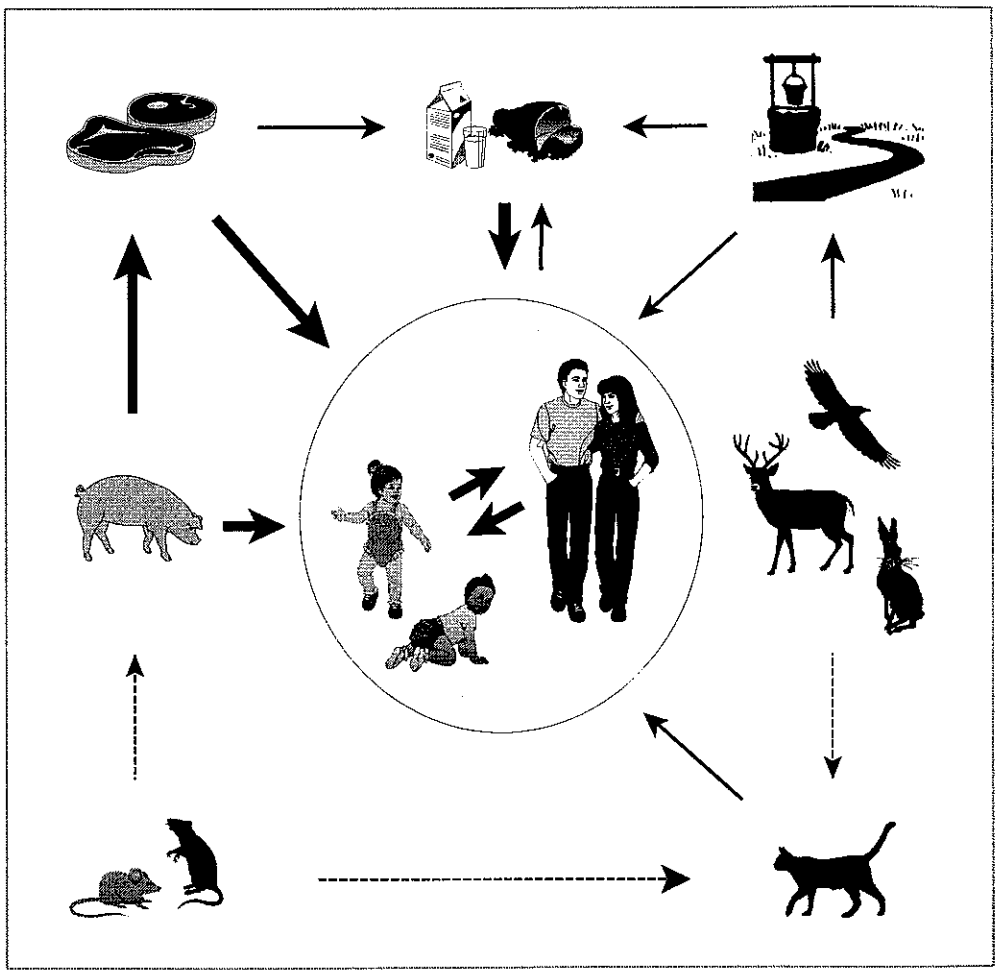


Figure 1.6 Transmission routes and recognized vehicles of *Y. enterocolitica*. The orientation and the heaviness of the arrows correspond with, respectively, the direction of the transmission and the level of documentation (heavy lines: well-documented; thin lines: rare; dotted lines: reasonable, but 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].

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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].

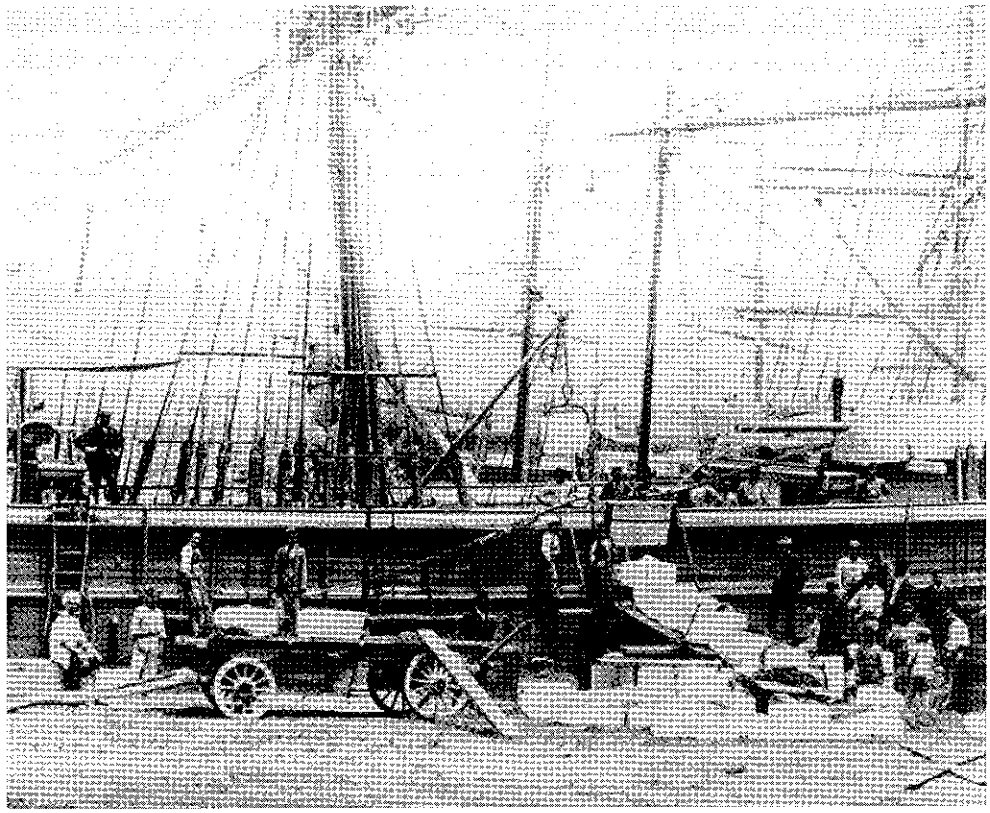

Figure 2.1 The sailing ship '*Tenax Propositi*' from Kragerø (Norway) in the harbour of Philippeville (Algeria), in 1886, delivering Norwegian ice. Copied from [Reinink & Vermeulen 1981], with permission.

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'

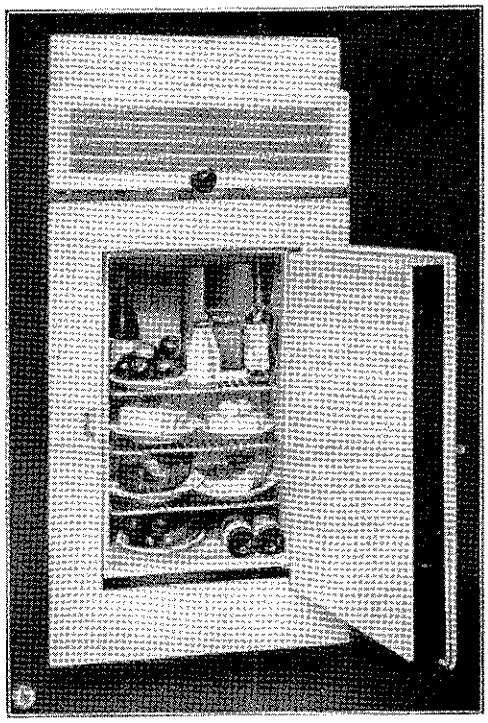

Figure 2.3 The 'Protos-Frigor': one of the first mechanical house-hold refrigerators in the Netherlands, (copied from *'Koeltechniek'*, 1932)

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.

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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).

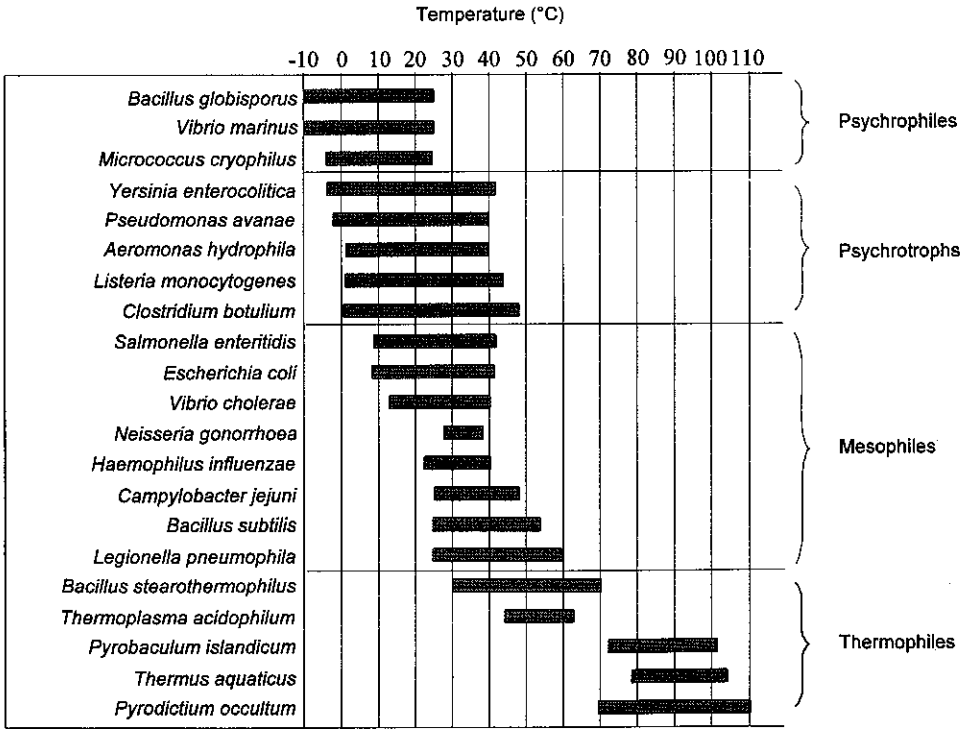

Figure 3.1 Growth temperature range of various bacteria, adapted from [Neidhardt *et al.* 1990]

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).

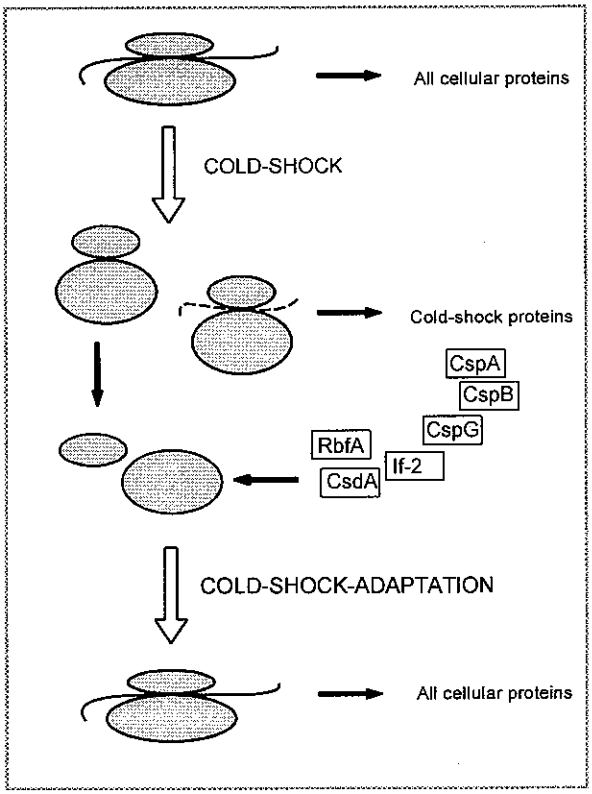

Figure 3.2 The Cold Shock Ribosomal Adaptation model, adapted from [Jiang *et al.* 1996b]

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.

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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).

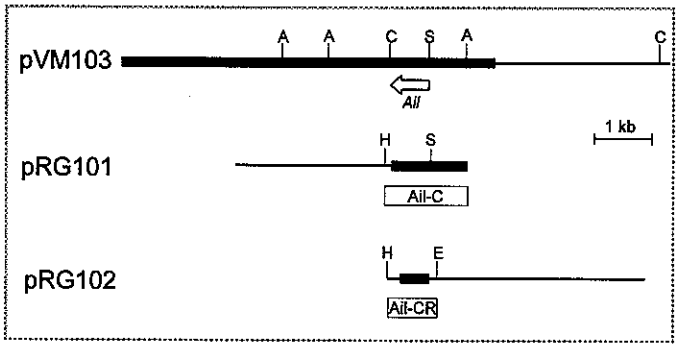

Figure 4.1
Plasmids and probes which harbour *ail*-sequences from *Y. enterocolitica*.
Bold lines: *Y. enterocolitica* DNA, thin lines: vector DNA. The position of the *ail*-gene is indicated by an arrow. Probes are depicted as open boxes. Relevant restriction sites are: A = *Ava*I, C = *Cla*I, E = *Eco*RI,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

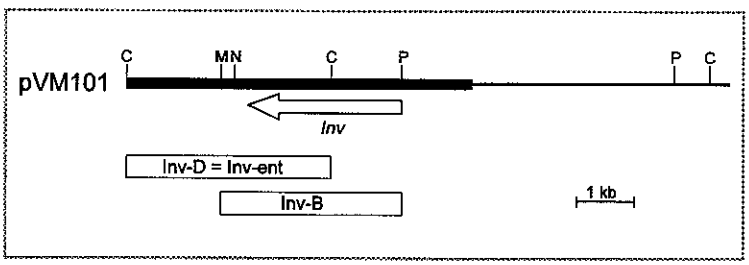

Figure 4.2
Plasmid and probes which harbour *inv*-sequences from *Y. enterocolitica*
Symbols as used in Figure 4.1 Relevant restriction sites are: C = *Cla*I, M = *Mlu*I, N = *Nco*I, and P = *Pvu*I.

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

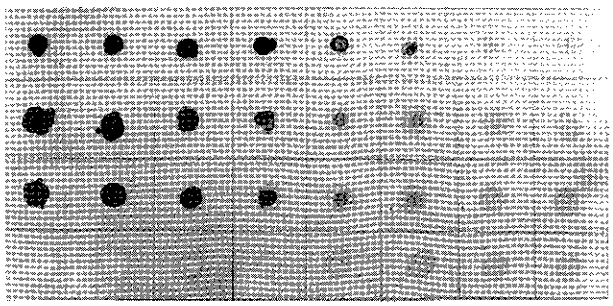

Figure 4.3
Inoculation scheme, and finished membrane, of a spot-blot-experiment. Spots contain 0.005-1 ng Ail-C DNA (as present in pVM103) or 2.5-5000 ng total genomic DNA of *Y. enterocolitica* (as purified from $5x10^5$ - 10^9 cells). The blot was hybridized with DIG-labelled Ail-C (1 ng/ml). Shaded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

O:9	O:3	O:9	O:3	O:3	O:9	O:9	O:9	O:3	O:3
O:9	O:3	O:9	O:3	O:3	O:9	O:9	O:3		O:9
O: 7,13	O: 6,30	O: 41,43	O:5	O: 6,31	O: 10,34	O: 6,31	O:5	O: 10,34	O: 12,25
pVM 101	*pVM 103*	*Y. int*	*Y. int*	*Y. pstub*	*Y. pstub*	*Y. fred*	*Y. fred*		

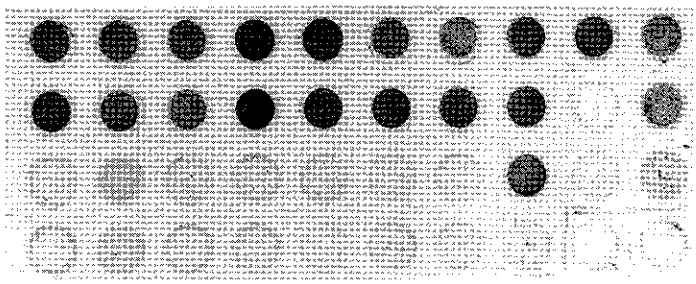

Figure 4.4
Inoculation scheme of a culture dot-blot containing various Yersinia species, and finished membrane after hybridization with DIG-labelled Ail-C (15 ng/ml). Each dot contained DNA from ca. 10^8 cells. Shaded inoculation sites indicate that the respective strains were presumed to contain *ail* DNA (these were.: TCI$^+$ *Y. enterocolitica*, and *Y. pseudotuberculosis*). The numbers refer to the serotypes of the strains of *Y. enterocolitica*. Positive and negative con-trols were, respectively, *E. coli* harbouring pVM103 and *E. coli* harbouring pVM101.Three wells were left empty.

Specificity of *ail*-probes in colony-blots

Next, colony blots of 63 *Y. enterocolitica* strains of known TCI-phenotype were hybridized with Ail-CR, and again a fair correlation was observed between serogroup, TCI-phenotype and hybridization with *ail*-probes (Table 4.2).

Table 4.2 Correlation between serogroup, *in vitro* invasiveness, and hybridization with *ail*-probes in culture dot-blots or colony blots of various strains of *Y. enterocolitica*

SEROTYPES	INVASIVENESS[1]		CULTURE HYBRIDIZATION[2]		COLONY HYBRIDIZATION[2]	
	+	-	+	-	+	-
O:3, O:9	11	0	11	0	11	0
O:5	6	8	6	8	6	8
various[3]	0	38	2[4]	36	2[4]	36
total number of strains	17	46	19	44	19	44
	63		63		63	

[1] Results in a Tissue Culture Invasion assay [Miller & Falkow 1989]: + = invasive, - = non-invasive.
[2] Hybridization signals as obtained with *ail*-probes: + = hybridization, - = no hybridization.
[3] Serotypes commonly not associated with disease, including O:4,33 /6,30 /6,31 /7,8 /7,13a,b /10,34 /12,25 /14 /16a,b /16,58 /18,49,50 /27,41,43 /36 /40 /41,43 and O:47.
[4] Strains of serotype O:4,33 and O:6,30.

No differences in the results were observed when blots were taken from either PCA, TSB or SSDC agar plates, or after incubating the plates at 37°C instead of 22°C (results not shown). An example of a finished colony blot membrane is given in Figure 4.5.

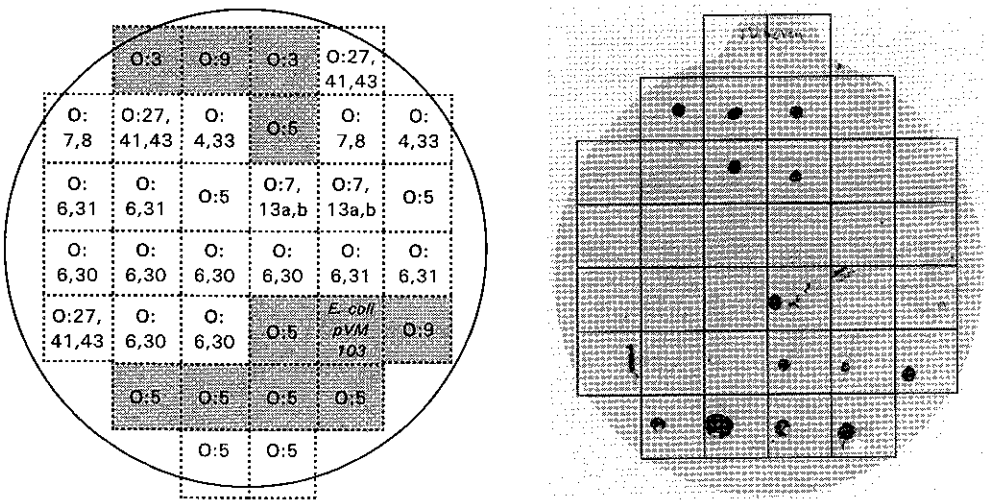

Figure 4.5 Inoculation scheme of a colony-blot containing various strains of *Y. enterocolitica*, and finished membrane after hybridization with DIG-labelled Ail-CR (20 ng/ml). The figures in the scheme refer to the serotypes of the strains, and shading of inoculation sites corresponds with *in vitro* invasiveness (TCI^+ strains). *E. coli* harbouring pVM103 was used as a positive control.

Specificity of *ail*-probes in Southern blots

Finally, six strains, including two TCI^+/ail^+ (serotype O:5), two TCI^-/ail^- (serotypes O:5 and O:7,8) and the two aberrant TCI^-/ail^+strains (serotypes O:4,33 and O:6,30) were analyzed by Southern blotting. After digestions with either *Eco*RV/*Sca*I, *Sau*IIIA1 or *Sau*IIIA1/*Cfo*I, the TCI^+ O:5-strains produced, *ail*-hybridizing fragments of 7.2, {1.7 + 0.7}, and 0.75 kb, respectively. TCI^- strains of serotype O:5 and O:7,8 did not hybridize. The two anomalous TCI^- strains showed identical hybridization patterns, comprising hybridizing fragments of 2.8, 4.6 and 0.5 kb, respectively (data not shown).

The observed sensitivity and specificity indicated that both the culture dot-blot and colony blot hybridization method could be used for a reliable discrimination between pathogenic and non-pathogenic *Y. enterocolitica*. However, false positive signals cannot be excluded since non-invasive strains might harbour non-functioning *ail*-genes.

Specificity of *inv*-probes

Hybridizations with Inv-B and Inv-D were performed on colony blots bearing several *Yersinia* species, type strains of other invasive species (*E. coli* O:128, *S. typhimurium*, *S. enteritidis*, and *L. monocytogenes*) and the *E. coli* hosts of pVM101, pVM103, pRG101 or pRG102. The results are presented in Table 4.3; an example of a 'finished' membrane is in Figure 4.6.

Table 4.3 Hybridization of various bacterial species with *ail*- and *inv*-probes which were derived from *Y. enterocolitica*

BACTERIAL SPECIES	HYBRIDIZATION[1]		
	Inv-B	Inv-D	Ail-CR
E. coli DH5α harbouring pVM103	±	-	+
E. coli DH5α harbouring pRG101	-	-	+
E. coli DH5α harbouring pRG102	±	-	+
putative pathogenic *Y. enterocolitica*[2]	++	++	++
Y. pseudotuberculosis	±	++	-
E. coli DH5α harbouring pVM101	++	+	-
putative non-pathogenic *Y. enterocolitica*[3]	+	+	-/+[4]
Y. intermedia	+	+	-
Y. frederiksenii	±	+	-
E. coli serotype O:128	+	+	-
S. enteritidis phage type 1	-	-	-
S. enteritidis phage type 2	-	-	-
S. typhimurium phage type 20	-	±	-
S. typhimurium phage type 150	-	±	-
L. monocytogenes serotype 1/2A	-	++	-
L. monocytogenes serotype 4B	-	-	-

[1] Signals obtained in hybridizations with *inv*- and *ail*-specific probes, as described in this work. - = no hybridization; ± = weak, + = moderate, and ++ = strong hybridization.
[2] Seventeen invasive (TCI^{+}) strains (serotypes O:3, O:9 or O:5).
[3] Forty-four non-invasive (TCI^{-}) strains (serotypes O:6,30 /6,30 /7,8 /10,34 /12,25 /13a,b /14 /16,58 /27,41,43 and O:36).
[4] + were two TCI^{-} strains of serotypes O:4,33 and O:6,30.

All *Y. enterocolitica* strains tested, as well as the control strain (*E. coli* harbouring pVM101), produced strong positive signals with both probes. Other *Yersinia* species produced moderate to weak signals. Surprisingly, weak positive signals with Inv-B were also obtained from *E. coli* strains harbouring pVM103 or pRG102, but these might be due to the presence of small amounts of vector DNA, that could be co-purified with the Inv-B fragment. Furthermore, both probes also produced a clearly positive signal with *E. coli* O:128. In addition, Inv-D also produced a strong hybridization signal with *L. monocytogenes* serotype 1/2A and a weak signal

with *S. typhimurium* (both phage types). These positive signals suggested the presence of *inv*-related genes in these species. Otherwise, the unexpected positive hybridization signals might be due to the long flanking sequences in the *inv*-probes, especially in probe Inv-D.

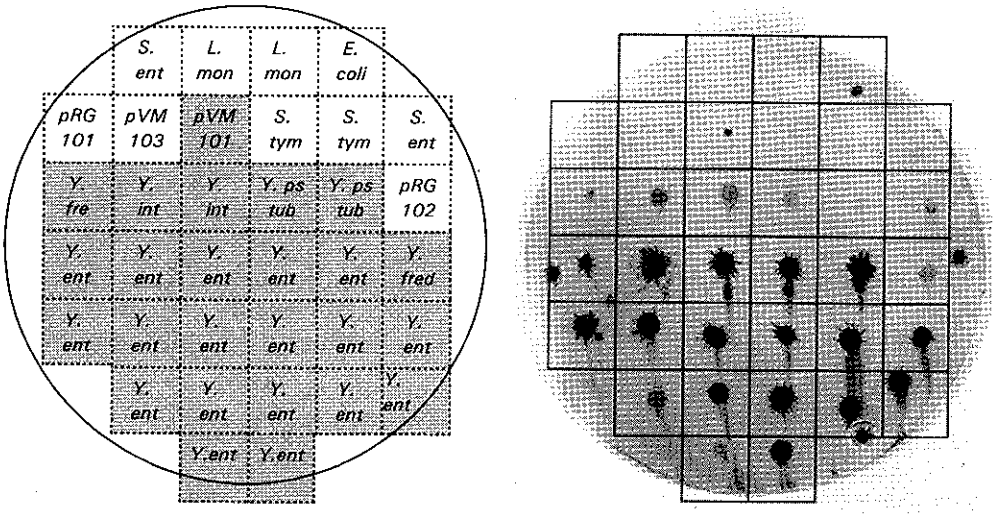

Figure 4.6 Inoculation scheme of a colony blot bearing several bacterial species, and finished membrane after hybridization with a DIG-labelled Inv-B -probe (20 ng/ml). Strains used were: *S. ent* = *Salmonella enteritidis*, *S. tym* = *Salmonella typhimurium*, *L. mon* = *L. monocytogenes*, *Y. fred* = *Yersinia frederiksenii*, *Y. int* = *Y. intermedia*, *Y. pstub* = *Y. pseudotuberculosis*, and *Y. ent* = *Y. enterocolitica*. Inoculation sites of strains that were presumed to contain *inv* sequences are shaded. Positive and negative controls were, respectively, *E. coli* DH5α harbouring pVM101 and *E. coli* DH5α harbouring pVM103, pRG101 or pRG102.

4.3.3 Analysis of Naturally Contaminated Samples

Pigs' organ samples

Mixed floras on selective agar plates were obtained from fourteen samples of pig tongues. On each plate, five suspect colonies, which resembled *Yersinia* on the basis of their morphology, were marked and partly removed for subculturing and identification. Colony blots were taken from the original plates and hybridized with Ail-CR. An example of a finished membrane is given in Figure 4.7, and the results are presented in Table 4.4.

All suspect colonies which had been identified as *Y. enterocolitica* and biochemically classified as presumably pathogenic (biotype 4, serotype O:3) appeared to be positive by hybridization.

Table 4.4 Detection of *Yersinia*-like colonies in primary isolates of pig tongues.

AMOUNT OF SAMPLES	ISOLATION METHOD[1]	BIOCHEMICAL IDENTIFICATION[2]	IDENTIFICATION BY HYBRIDIZATION[3]
8	SSDC	*Y. enterocolitica* O:3	+[4]
1	SSDC	*Y. enterocolitica* O:6,30	-
1	SSDC	not *Yersinia*[5]	-
1	SSDC	*Y. enterocolitica* O:3 & *Y. enterocolitica* O:5	+ -
1	SSDC CIN	*Y. enterocolitica* O:3 not *Yersinia*[5]	+ -[5]
1	CIN	*Y. enterocolitica* O:3	+
1	CIN	not *Yersinia*[5]	-

[1] Isolation on SSDC and/or CIN-agar, as described elsewhere [De Boer *et al.* 1989].
[2] Identification and serotyping of typical colonies by established methods, as described elsewhere [Bercovier *et al.* 1980; Wauters *et al.* 1987].
[3] Signals obtained in hybridization with probe Ail-CR, as described in this paper. + = hybridization, - = no hybridization.
[4] shown in Figure 4.7: middle and right side parts of the mebrane.
[5] shown in Figure 4.7: left side blot part of the membrane.

In contrast, no hybridization was observed with colonies which had been identified as presumably non-pathogenic *Y. enterocolitica* (serotypes O:5 and O:6,30, biotype 1), or as non-*Yersinia* species.

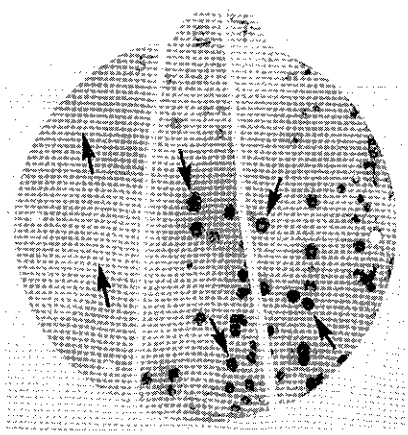

Figure 4.7
Finished membranes of colony blots taken from primary isolates of pig tongues, after hybridization with DIG-labelled Ail-CR (30 ng/ml).
One NEF-978X membrane was cut into three pieces and used for colony lifts from three different samples. The colonies that were subcultured are marked with arrows. The subcultured colonies from the left side blot were biochemically classified as not belonging to the genus Yersinia. All subcultured colonies from the two other blots were identified as *Y. enterocolitica* serotype O:3.

These results indicated that identification of pathogenic *Y. enterocolitica* by hybridization with *ail*-probes was at least as specific as identification by conventional methods.
Next, mixed flora on selective agar plates were obtained from thirteen samples of pigs' intestinal organs, collected in a slaughterhouse. From each plate, five morphologically *Yersinia*-like colonies were subcultured and biochemically identified. Eleven colony blots, taken from the original agar plates, were cut in two pieces and analyzed with both Ail-CR and Inv-D. Two blots were hybridized with Inv-D only. The results are presented in Table 4.5.
Y. enterocolitica serotype O:5,27 could be isolated from two samples, and only these samples were positive by hybridization with the *inv*-probe. One of these samples was also examined for hybridization with *ail*, and produced a positive signal in this test.

Human faecal samples

Apparently homogenous floras on selective agar plates were obtained from six samples of human diarrhoeal faeces. From each plate, several *Yersinia*-like colonies were subcultured, identified and serotyped. Colony blots from the original plates were hybridized with Ail-C. Only one sample was positive by hybridization, while *Y. enterocolitica* serotype O:3 was isolated from this sample by subculturing (Table 4.5). No hybridization was observed with plates from which other *Yersinia* serotypes, or non-*Yersinia* species, had been isolated. The results were identical for all described isolation procedures.

Slaughterhouse swab-samples

Mixed flora on selective agar plates were obtained from 53 slaughterhouse swab-samples. From each primary isolate, five *Yersinia*-like suspect colonies were subcultured and identified biochemically. Colony blots were taken from the original plates, cut in two pieces, and hybridized with *ail*- and *inv*-probes. The results are also presented in Table 4.5.
Most of the swab-samples (87%) showed the expected correlation: i.e. positive hybridization signals when *Y. enterocolitica* was isolated and no hybridization when *Y. enterocolitica* was not found in the sample. The data included (i) two samples from which one ore more pathogenic *Y. enterocolitica* were isolated and from which the blots were positive for both *ail* and *inv*; (ii) 44 samples that were negative by biochemical analysis as well as by hybridization; (iii) two samples which were negative by biochemical analysis although they produced clearly positive signals with the *inv*- and/or *ail*-probe; and (iv) five samples that produced (very) weak positive signals with Ail-CR and/or Inv-B, while *Y. enterocolitica* could not be isolated from the corresponding plates. These results suggested that, for this type of sample, our method is probably more sensitive in detecting *Y. enterocolitica* then are conventional methods.
Finally, two isolates were arbitrary chosen from subcultured suspect colonies which could not be identified as *Yersinia*, and further identified by a semi-automated analysis system (Titertek Enterobacteriacae Automated System, TTE-AS, Flow Laboratories, Irvine, Scotland). It appeared that these isolates belonged to the species *Kluyvera cryocrescens*. These isolates were also tested in colony blot hybridizations, and produced - to our surprise - strong positive

signals with both *ail*- and *inv*-probes (results not shown). However, the two isolated *Kluyvera* strains originated from plates that had produced negative colony blots.

Table 4.5 Detection of *Y. enterocolitica* by biochemical or DNA-hybridization methods, in samples of human, porcine or slaughterhouse origin[1].

SAMPLE TYPES AND NUMBERS		BIOCHEMICALLY IDENTIFIED COLONIES[2]	HYBRIDIZATION[3] *ail*	*inv*
Human faeces	1	*Y. enterocolitica* O:3	+	nm
Human faeces	1	*Y. enterocolitica* O:16a,58	-	nm
Human faeces	1	*Y. enterocolitica* O:6,31	-	nm
Human faeces	3	other genera	-	nm
		amounts tested	6	0
Pig tongue	2	*Y. enterocolitica* O:5,27	+/nm	+
Pig tongue	6	other genera	-	-
Pig rectum	3	other genera	-/nm	-
Pig ileum	2	other genera	-	-
		amounts tested	11	13
Swab-sample	1	*Y. enterocolitica* O:3	+	+/-[4]
Swab-sample	1	*Y. enterocolitica* O:3	+/-[4]	+/-[4]
Swab-sample	44	other genera	-	-
Swab-sample	1	other genera	-	±
Swab-sample	1	other genera	-/±[4]	-
Swab-sample	1	other genera	+	+
Swab-sample	1	other genera	+	-
Swab-sample	2	other genera	±	±
Swab-sample	1	other genera	±	-
		amounts tested	53	53
total samples	72	total amounts tested	70	64

[1] Samples were human diarrhoeal stools, (contents of) pigs' gastro-intestinal organs, and swabs taken from pig carcasses, from machinery or from employees hands in a pig slaughterhouse.

[2] Isolation, identification and serotyping as described elsewhere [de Boer *et al.* 1989; Bercovier *et al.* 1980; Wauters *et al.* 1987, respectively]. Organisms which could not be classified as *Yersinia* species were not identified to the species level.

[3] Signals obtained in colony blots using *Yersinia*-derived probes, as described in this paper. - = no hybridization, ± = weak, and + = strong hybridization.

[4] Signals obtained with colony blots from CIN or SSDC-plates, respectively.

4.4 DISCUSSION

Tissue Culture Invasion

The results of a tissue culture invasion-assay with 132 serotyped European strains showed a 100% correlation between invasiveness and serotypes associated with disease. This was in agreement with the results obtained with American strains [Miller & Falkow 1988; Miller *et al.* 1989] and strongly supported the assumption that the TCI^+ phenotype corresponds with *in vivo* pathogenicity. Hence, we used the TCI phenotypes to designate a strain as pathogenic (TCI^+) or non-pathogenic (TCI^-).

Sensitivity of Digoxigenin-labelled Probes

As non-radioactive marker for the probes, we used a commercially available kit employing digoxigenin (DIG). In the course of our experiments, several modifications were introduced in the original hybridization protocol as provided by the manufacturer. These modifications presumably resulted in a more effective blocking of non-specific binding sites on the membrane, because we observed improvements with regard to reproducibility, membrane-preservation and signal-to-noise ratio at the desired level of sensitivity. The lower detection limit of purified Ail-C-homologous DNA, as measured in a 1 µl spot-blot assay with Ail-C as a probe and applying the modified protocol, ranked between 0.001 and 0.005 ng, when present in immobilized pVM103. The detection limit of Ail-C-homologous DNA in the total genomic matrix. from TCI^+ *Yersinia* cells ranged between 5 to 25 ng of genomic DNA, setting the detection limit at $5x10^6$ cells. In contrast, up to 1000 ng of DNA from TCI^- cells only produced background signals. These results indicated that DNA from to 10^9 TCI^+ cells could be clearly distinguished from corresponding amounts of DNA out of TCI^- cells.

Specificity of Digoxigenin-labelled Probes

The specificity of DIG-labelled *ail*-probes was studied on culture dot-blots and colony blots derived from strains of known TCI phenotype. Two *ail*-fragments were used, Ail-C and Ail-CR, from which the Ail-CR-probe was expected to be more specific, since it hardly contains flanking sequences [Miller *et al.* 1989, Miller *et al.* 1990]. All 39 TCI^+ strains tested gave a positive hybridization signal in the culture dot-blot and colony blot experiments, confirming previous observations [Miller & Falkow 1988] that a TCI^+ phenotype strongly correlates with the presence of the *ail*-gene. No differences were observed between colony blots or culture dot-blots, nor between the Ail-C or Ail-CR probe. Thus, the observed specificity showed that DIG-labelled Ail-C and Ail-CR-probes were equally well suited for discrimination between pathogenic and non-pathogenic strains of *Y. enterocolitica* in both culture dot-blot and colony blot hybridizations. Only two out of 174 non-pathogenic strains (serotypes O:4,33 and O:6,30] gave a positive hybridization signal with Ail-probes. Southern blot analysis of these two TCI^- /ail^+ strains revealed hybridization patterns that were at variance with those from TCI^+ strains. Presumably, the anomalous strains harbour a non-functional *ail*-gene. Future DNA-sequencing

could reveal whether this is due to insertions or deletions in or near the *ail*-gene.
The specificity of hybridization with DIG-labelled *inv*-probes was studied on colony blots of several species, using two *inv*-fragments, Inv-B and Inv-D. Because of its closer similarity with the pure *inv*-gene [Young *et al.* 1990], Inv-B was expected to be more specific than Inv-D. Although all *Y. enterocolitica* strains were clearly positive by hybridization with both Inv-B and Inv-D, the other *Yersinia* species only produced weak to moderate signals. Furthermore, positive signals were observed with *E. coli* O:128, *L. monocytogenes* and *S. typhimurium*, but these results might be due to the presence of sequences flanking *inv*, especially in the Inv-D probe. In conclusion, the observed specificity with both probes was less pronounced than was previously reported [Miller *et al.* 1989]. Nevertheless, since Inv-B principally hybridized with all *Y. enterocolitica* and with only one of the unrelated species tested, we concluded that Inv-B is suitable for detection of *Y. enterocolitica* in a mixed bacterial flora.

Correlation of Hybridization and Biochemical Analysis

The reliability of the method for detection of pathogenic and non-pathogenic *Y. enterocolitica*, using, respectively, DIG-labelled *ail*- and *inv*-probes, was tested on naturally contaminated samples. The results were in good agreement with the results of conventional biochemical tests. We have found that (i) all colonies obtained from pig tongues which were identified as pathogenic *Y. enterocolitica* on the basis of their serotypes (eleven strains) hybridized with the *ail*-probe, whereas strains of other serotypes (two strains) did not; (ii) the only two samples of human faeces and pigs' organs from which pathogenic serotypes were isolated also hybridized with the *ail*-probe, and a sample that contained a non-pathogenic serotype hybridized with the *inv*-probe, while other samples did not hybridize; and (iii) the only two slaughterhouse swab-samples from which pathogenic serotypes of *Y. enterocolitica* were isolated were also positive in hybridization with *ail*. On the other hand, moderate to strong signals with both *inv*- and *ail*-probes were also obtained from several samples in which no Yersinias could be detected by biochemical analysis. False positive results with the *inv*-probes might be caused by the presence of enterotoxigenic strains of *E. coli*, since we had observed that *inv*-probes can also hybridize with *E. coli* O:128. This cross reaction might be the result of interference with the *E. coli eae* gene, because the product of this gene shows significant homology with invasin, the product of the *Y. pseudotuberculosis inv* gene [Jerse *et al.* 1990]. Although we did not observe cross-reactions of *ail*-hybridization with other entero-invasive bacterial species, false positive signals with *ail*-probes might be caused by the presence of *S. typhimurium*, because its *pagC* locus shows similarity with the sequence of *ail* [Pulkkinen & Miller 1991]. However, no correlation was found between the 'false positive' signals obtained with *ail*-probes and the isolation of Salmonella strains from several samples. Finally, *K. cryocrescens* may represent another source of false positive results. This flagellated, facultatively anaerobic, Gram-negative organism, which may be an opportunistic pathogen of man [Farmer 1984], was isolated from several slaughterhouse swab-samples and the isolates appeared to hybridize with both *ail* and

inv. However, no hybridization had been found with colony blots from the corresponding plates. This suggested that the original plates contained trace amounts, i.e. below the detection limit, of *K. cryocrescens* which overgrew the initially dominant species of the subcultured colonies in the subsequent subculturing steps on less selective media.
In a few samples, we observed differences between the results of colony blots obtained from SSDC or CIN plates. In most of these cases, the CIN plate signals showed a better correlation with the results of biochemical detection. Possibly, ingredients in the medium diffused from the SSDC-agar into the membrane and interfered with the DNA-fixation or the hybridization.

Practical Significance

In previous studies, using [^{32}P]-labelled probes derived from *inv* and *ail* [Miller & Falkow 1988], *inv* was shown to be specific for the genus *Yersinia*, whereas *ail* appeared to be restricted to *Yersinia* strains commonly associated with disease [Miller *et al.* 1989; Robins-Browne *et al.* 1989]. The aim of our study was to develop a method based on non-radioactive labelled probes derived from *ail* and *inv*, and to use this method for distinguishing between pathogenic and non-pathogenic *Y. enterocolitica* strains and for rapid detection of *Yersinia* in samples of human or animal origin.
Recently, it was suggested that the exclusive use of conventional isolation procedures may well lead to underestimation of pathogenic *Y. enterocolitica* in naturally contaminated pork products [Nesbakken *et al.* 1991], an assumption which was based on the results of a comparative study of several detection methods. Although we did not find a large discrepancy between the results of biochemical methods and hybridization, our results indicate that detection based on DNA-hybridization is more sensitive than the conventional analysis.
Our results demonstrate that, by following the described modifications of the hybridization protocol, DIG-labelled *ail*-derived probes can be used for: (i) the discrimination between pathogenic and non-pathogenic *Y. enterocolitica* strains; and (ii) the detection of pathogenic *Y. enterocolitica* in primary isolates from natural samples. Likewise, *inv*-derived DIG-labelled probes can be used to detect *Yersinia* species in natural samples. A risk of false positive results can not be excluded totally, because genes related to *ail* or *inv* may be present in other species.

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5

Temperature & Phenotype

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Growth rate and physiology of *Yersinia enterocolitica;* influence of temperature and presence of the virulence plasmid

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ABSTRACT

The effect of temperature on the growth rate, protein pattern and fatty acid composition of *Yersinia enterocolitica* strain W22703 PYV$^+$, its plasmidless isogenic derivative W22703 pYV$^-$, and four recent field isolates was examined.

The growth rate was clearly influenced by the presence or absence of the virulence plasmid: pYV$^-$ strains grew consistently faster than pYV$^+$ strains. This difference in growth rate was moderate to high at 25-35°C and at 1-10°C, but hardly significant at 15-20°C. Increase of the growth temperature to 25°C and above, resulted in the induction of the 220 kDa virulence plasmid-encoded YadA protein. Between 1-20°C, by contrast, no obvious temperature- or plasmid-related differences in protein patterns could be detected. With respect to the fatty acid composition, a clear temperature-dependent change was observed: with all strains, the degree of saturation was low at 1°C, but gradually increased with raising temperatures. All strains, except one field isolate, had similar fatty acid patterns. The presence or absence of the virulence plasmid in W22703 did not significantly alter the fatty acid pattern, but one of the field isolates showed aberrant C16:1 and cyclic fatty acid contents in the 5-25°C and ≤15°C ranges, respectively.

5.1 INTRODUCTION

Yersinia enterocolitica is a psychrotrophic, facultatively anaerobic bacterium, which has regularly been implicated in food-borne infections. It is frequently present in foods of animal origin and epidemiological studies reveal a coincidence of outbreaks of yersiniosis and consumption of such contaminated foods [Andersen 1988; Kapperud 1991]. Refrigerated foods are commonly potential vehicles for growth of *Y. enterocolitica*, as it can grow at low temperatures. This psychrotrophic character possibly accounts for the increased incidence of the disease in the last five decades, as refrigeration has gradually replaced traditional methods of domestic and industrial food preservation.

During the 1970s, characterization of Yersiniae, occurrence, growth potential and pathogenicity to humans has been the subject of many studies [reviewed by: Hurvell 1981; Lee 1977; Stern & Pierson 1979; Zink *et al.* 1982; Swaminathan *et al.* 1982]. Several different serotypes may be distinguished in the species *Y. enterocolitica*, but only few of them, especially O:3, O:8, and O:9, are virulent to humans. Molecular biological studies have shown that pathogenicity correlates both with the presence of a virulence plasmid (pYV) and the presence of a chromosomal adhesion-invasion-locus (*ail*) [Portnoy & Martinez 1985; Cornelis *et al.* 1987; Isberg 1989; Miller 1992]. The pYV plasmid contributes to virulence by encoding for a set of proteins called Yops (Yersinia Outer membrane Proteins) which are produced above 30°C only. This temperature-dependent production of Yops is attended by severe growth restriction, and the process is mediated by the pYV-encoded transcriptional activator virF, which in turn is regulated by the chromosomally-encoded protein YmoA [Cornelis *et al.* 1989]. Apart from temperature, other environmental factors, such as Ca^{++}, also influence the synthesis of Yops. Loss of pYV leads to colony dimorphy, as is frequently observed at 37°C [Berche & Carter 1982; Lazere & Gemski 1983; Bhaduri *et al.* 1990]. In addition to the plasmid-mediated synthesis of Yops, several chromosomal encoded features of *Y. enterocolitica* are also dependent on the growth temperature. When grown below 30°C, the bacterium becomes motile due to the production of peritrichous flagella [Bercovier & Mollaret 1984], and the bacterial cell surface hydrophobicity increases due to the onset of fimbrial protein synthesis [MacLagan & Old 1980; Old & Robertson 1981; Faris *et al.* 1983; Skurnik 1984]. In addition, there is also an effect of growth temperature on the lipopolysaccharide (LPS) synthesis: the bacterium produces a 'smooth'-type of LPS when grown below 25°C and a 'rough'-type LPS at higher temperatures [Portnoy & Martinez 1985].

In view of its psychrotrophic nature, the frequent occurrence of *Y. enterocolitica* on nutritional resources, such as freshly-slaughtered pig carcasses, presents a serious problem in maintaining food safety [Andersen 1988]. Several studies have been devoted to the effects of temperature on the growth of this organism [Buckeridge *et al.* 1980; Kendall & Gilbert 1980; Stern *et al.* 1980a,1980b; Brocklehurst & Lund 1990; Little *et al.* 1992], but none addresses how *Y. enterocolitica* obtains its broad temperature growth range.

It is known that one of the commonest adaptations of bacteria to varying growth temperatures is an alteration in the fatty acid composition of their membrane lipids, in order to maintain a proper degree of membrane fluidity [McElhaney 1976; Russell 1984]. This thermal adaptation has been shown for numerous organisms, including *Escherichia coli* [Marr & Ingraham 1962; Cronan & Vagelos 1972] and *Y. enterocolitica* [Abbas & Card 1980]. The pattern usually observed is a shift to higher contents of long-chain, saturated fatty acids with increasing growth temperature. As a first attempt to unravel the factors that could be responsible for the ability to grow in the cold, we studied the bacterial growth rate and the cellular protein- and fatty acid compositions in a wide temperature range (1-35°C), as well as the influence of pYV on these characteristics.

5.2 MATERIALS & METHODS

5.2.1 Bacterial Strains

The *Y. enterocolitica* reference strain W22703 pYV^+ (serotype O:9), and its isogenic plasmid-cured variant W22703 pYV^-, were obtained from Prof Dr G. Cornelis (Université Catholique de Louvain, Bruxelles, Belgium). Wild-type strains of human origin (nrs. 89.029 and 89.430, serotypes O:9 and O:6,30, respectively) were obtained from the National Institute of Public Health and the Environment (RIVM), Bilthoven, the Netherlands. Strains of porcine origin (nrs. 6901 and 6907, serotypes O:3 and O:41,43, respectively) were collection strain at our institute. Strains were stored at –70°C in nutrient broth with 25% (v/v) glycerol. Fresh cultures were prepared by incubation in Brain Heart Infusion (BHI, Oxoid) at 30°C for 18-20 h.

5.2.2 Determination of Growth Characteristics

Growth on solid phase

Fresh cultures were prepared from the strains to be tested, and bacterial concentrations were estimated by measuring the optical density at 600nm (OD_{600}). Appropriate dilutions in 0.9% NaCl were spread on Standard Plate Count Agar (PCA, Oxoid) plates, yielding 10-100 colonies per plate. Plates were incubated at 1, 5, 10, 15, 20, 25, 30 or 35°C. At regular intervals, starting at 17 h, mean colony-diameters were measured with a 25x magnification microscope equipped with a Glarex projection head (Carl Zeiss, Jena, Germany). Incubation was discontinuated when no further increase of colony-diameters could be detected in three successive measurements.

Growth in liquid phase

RPMI-1640 broth (Gibco/BRL Life Technologies), temperature-equilibrated at 1, 10, 20 and 30°C, was 1:100 inoculated with appropriate dilutions of fresh cultures, to give initial

concentrations of 1-10 viable bacteria per ml. The inoculated media were placed on shaking platforms and incubated at 1, 10, 20 and 30°C. Samples were taken periodically, to record the growth, either by measuring the OD_{600} and/or the amount of colony forming units per ml (CFU). From the successive CFU-counts, growth curves were plotted, and the lag time, the specific growth rate, and the maximum concentration were calculated, using a non-linear regression fitting program [Zwietering *et al.* 1990]. Incubation was discontinued as soon as the cultures reached the late exponential phase, i.e. at $OD_{600} = 0.6$-0.8. Next, the cells were pelleted by low speed centrifugation, and cell-free supernatants were obtained by filtering the supernatants through a 0.2 µm filter (Acrodisc, Gelman Sciences, Ann Arbor, USA). The bacterial pellets were washed once with 0.9% NaCl. Washed cells and cell-free supernatants were stored at –20°C untill further use.

5.2.3 Plasmid-screening

The finished cultures of the growth experiments were assayed for potential loss of the virulence plasmid by subculturing and subsequent DNA hybridization. Briefly,appropriate dilutions of the RPMI-1640 cultures, as well as small inocula of full-grown colonies on the PCA-plates, were spread on fresh PCA plates and incubated at 30°C for 48 h, in order to obtain single colonies from individual cells. Next, ten randomly chosen single colonies from each secondary plate were both spotted onto hybridization membrane (Hybond-N, Amersham/Life Sciences) and inoculated into BHI broth. The membrane was placed on PCA agar and incubated at 30°C for 48 h, in order to obtain visible colonies. In these colonies, the presence or absence of pYV was determined by colony hybridization, using pYV(isolated from strain 89.029) as a probe. From the inoculated broth, the plasmid contents was isolated after overnight incubation at 30°C, and visualized using gelelectrophoresis. Standard techniques were applied for plasmid isolations, for labelling of pYV with ^{32}P, for gelelctrophoresis, and for hybridization [Maniatis *et al.* 1982].

5.2.4 Fatty Acid and Protein Analysis

Fatty acid composition

The total lipid contents from approx. $2x10^{10}$ cells, grown in RPMI-1640, were isolated by extraction with chloroform/methanol (1:2, v/v), as described elsewhere [Kates 1986]. The solvents were evaporated under nitrogen flow and the extract was trans-esterificated with 5% H_2SO_4 (v/v) in methanol, as described elsewhere [Christie 1980]. The fatty acid methylesters were isolated by extraction with hexane, dried under nitrogen flow and finally dissolved in 100 µl of hexane. Fatty acid analysis was done by capillary GLC on a HP5880 gas chromatograph (Hewlett/Packard, Avondale, USA) equipped with a flame ionization detector, using helium as carrier gas. Samples of 1 µl were injected at split ratio 1:200 on a 50m x 0.25 mm (inner

diameter) fused silica, wall coated open tubular column with CP-Sil88 as stationary phase (Chrompack, Middelburg, the Netherlands). The injector and detector temperatures were maintained at 250°C and 280°C, respectively, and the column temperature was held at 150°C for 4 min and subsequently raised to 220°C at an increase rate of 4°C per min. The relative area percentages of the peaks in the chromatograms were determined by a HP1000 Lab Data System integrator (Hewlett/Packard).

The fatty acid methylesters were identified by comparison of their retention times with those of pure compounds and standard mixtures. Quantitative mixtures of straight chain fatty acid methylesters, including C12:0-C21:0 (both even and odd carbon numbers), C14:1-C18:1 (even carbon numbers, both cis- and trans-isomers) and C18:2 (cis/cis- and trans/trans-isomers), were obtained from Alltech. Various other fatty acid methylesters were purchased from Sigma. These included straight chains C11:0, C20:0 and C22:0; branched chains C15:0iso-C21:0iso; nine different cis- and trans- isomers of mono-unsaturated chains C17:1-C20:1; poly-unsaturated chains C18:3 and C18:4; and hydroxylated chains C16:O-2-OH and C18:0-2-OH A bacterial cell wall preparation, that contained methylesters of the cyclic fatty acids C17:0c and C19:0c, was obtained from Supelco.

Protein composition

Portions of bacterial cell pellets, harvested after growth in RPMI-1640 and containing ± $5x10^8$ cells, were resuspended in 40μl of lysis buffer {0.05 M Tris-HCl pH=8.0, 5% sodium dodecyl sulphate (SDS), 0.015 M 2-mercapto-ethanol}, and denatured by heating 10 min at 100°C. The samples were mixed with 20 μl 3x concentrated gel loading buffer {0.188 M Tris-HCl pH=6.8, 6% SDS, 15% 2-mercapto-ethanol, 30% glycerol, 0.03% bromphenol blue}, and the proteins separated by SDS-PAGE, as described elsewhere [Laemmli 1970]. The acrylamide concentrations in the stacking and separating gels were 4%, and 10-15%, respectively. Fractions of cell-envelopes and cytosol were obtained by sonication of cell suspensions, and subsequent partial membrane solubilization in the detergent Sarkosyl (Ciba-Geigy, Wehr/Baden, Germany), essentially as described by [Achtman *et al.* 1983.]. Samples were denatured and analyzed by SDS-PAGE, as described above.

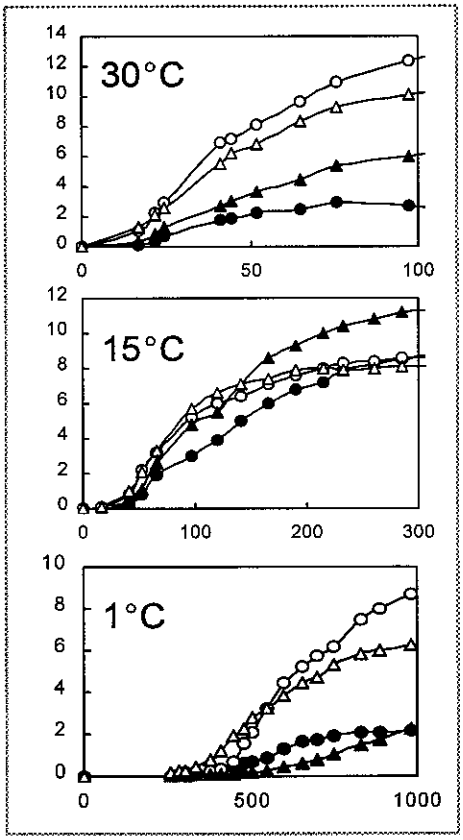
Cell-free supernatants originating from ± $2x10^9$ cells were 25x concentrated by centrifugal ultrafiltration using Microsep (Filtron, Northborough, USA) devices. The concentrates were mixed with 0.5 volumes of 3x concentrated gel loading buffer, and denatured as described above. The protein contents were separated by SDS-PAGE using 12% polyacrylamide gels. Gels were stained with Coomassie Brilliant Blue R250 or with silver (Silver Stain kit, Bio-Rad Corp., Richmond, CA, USA).

5.3 RESULTS

5.3.1 Growth Characteristics

Growth on Solid Phase

Preliminary incubations of wild-type *Y. enterocolitica* strains on PCA plates revealed that certain serotypes (i.e. O:9, O:3) needed a longer incubation period to produce visible colonies than others, i.e. 30 h at 30°C vs. 20 h at 30°C (results not shown). The slow growing strains were all pYV$^+$, which suggested that this phenomenon could be plasmid-related.



To study whether the incubation temperature affected this growth rate difference between plasmid-harbouring and plasmidless strains, two pYV$^+$ (89029, 6901) and two pYV$^-$ (89430, 6907) wild-type strains were incubated on PCA plates at different temperatures, ranging from 1 to 35°C, and colony-diameters were measured as a parameter of bacterial growth. At all temperatures, the increase of colony-diameters was slower with the plasmid-harbouring strains than with the plasmidless strains, but the differences were more pronounced at 1-10°C and at 30-35°C than at 15-25°C (Figure 5.1).

Figure 5.1
Growth rates of virulent (pYV$^+$) and non-virulent (pYV$^-$) *Yersinia enterocolitica* strains, grown on Plate Count Agar at optimal, medium or low temperature. On the x-axis is the incubation time (in hours), on the y-axis the colony-diameter (in mm). Virulent strains are serotypes O:9 (●) and O:3 (▲); non-virulent strains are serotypes O:6,30 (○) and O:41,43 (△).

In addition to these differences in growth rate, we observed also differences in maximum colony-diameter when incubations were continued over longer periods, up to 60 days (Table 5.1). Furthermore, at 30 and 35°C, colonies of the plasmid-harbouring strains sometimes became irregular shaped, due to the initiation of growth of secondary colonies on the rim of

the original ones (results not shown). Plasmid screening of the colonies at the end of the incubations showed that pYV was not lost at temperatures between 1 and 30°C. At 35°C, only one out of ten subcultured colonies of strain 6901 had lost its plasmid (results not shown).

Table 5.1 Maximum colony diameters (in mm) of *Y. enterocolitica* wild type strains, grown on Plate Count Agar at different temperatures

STRAINS		INCUBATION TEMPERATURE							
code	geno-, serotype	1°C	5°C	10°C	15°C	20°C	25°C	30°C	35°C
89.029	pYV+, O:9	3	3	11	9	10	3	3	3
6901	pYV+, O:3	3	9	11	12	9	12	10	8
89.430	pYV-, O:6,30	9	9	7	8	>14	>14	>14	14
6907	pYV-, O:41,43	7	7	10	8	10	9	12	12

Growth in Liquid Phase

The influence of the virulence plasmid on bacterial growth at different temperatures was further investigated in RPMI-1640. With the isogenic strains W22703 pYV^+ and W22703 pYV^-, significant differences in growth rate were observed at 1, 5 and 30°C, but not at 20°C (Figure 5.2). A similar pattern was observed with the four wild type strains, i.e. the growth rates of pYV^-strains exceeded those of pYV^+ strains much more at low (1-10°C) and high (25-35°C) temperatures than at intermediate temperatures (15 or 20°C) (Table 5.2).

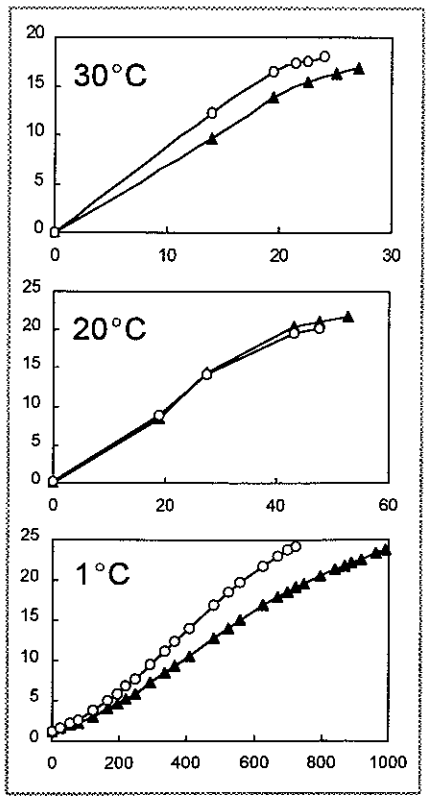


Figure 5.2
Growth rates of the pYV^+ *Y. enterocolitica* reference strain W22703 (▲), and its plasmidless (pYV^-) derivative ((O) in broth cultures at optimal, medium and low temperature. On the x-axis is the incubation time (in hours), on the y-axis is the increase in bacterial density (in logN/No).

Table 5.2 Growth rates μ (h^{-1}) of various strains of *Y. enterocolitica* in RPMI-1640 at various temperatures

T (°C)	pYV+ STRAINS			pYV- STRAINS			INCREASE/DECREASE μ pYV- vs. μ pYV+ strains
	89.029	6901	average	89.430	6907	average	
1	0.06	0.06	0.06	0.08	0.07	0.07	increase 22%
5	0.12	0.13	0.12	0.16	0.17	0.16	increase 32%
10	0.22	0.24	0.23	0.29	0.28	0.29	increase 25%
15	0.32	0.35	0.34	0.37	0.36	0.36	increase 8%
20	0.53	0.51	0.52	0.59	0.51	0,55	increase 5%
25	0.63	0.57	0.60	0.82	0.77	0.80	increase 33%
30	0.65	0.47	0.56	1.07	0.93	1.00	increase 79%
35	0.71	0.66	0.68	1.81	1.04	1.43	increase 109%
	pYV+ reference strain W22703			pYV- reference strain W22703			
1	0.030 ± 0.001			0.040 ± 0.003			increase 34%
5	0.123 ± 0.016			0.138 ± 0.021			increase 15%
20	0.770 ± 0.127			0.695 ± 0.264			decrease 10%
30	1.157 ± 0.145			1.290 ± 0.251			increase 12%

Plasmid loss was not detected in any of these strains, nor at any temperature (results not shown). Occasionally, colony dimorphy was seen on the CFU-counting plates of the pYV^+ strains. We observed, in addition to typical flat, white colonies (designated type I), sometimes also smaller, more convex and somewhat translucent colonies (designated type II) (results not shown). This phenomenon occurred mainly at 25-30°C and diminished after prolonged incubation. We presumed that this was due to loss of the plasmid in the larger colonies, but plasmid isolations from several subcultured small and large colonies revealed that both types still harboured pYV. Subculturing of small and large colonies on fresh PCA yielded only colonies of type I, which, again, all appeared to harbour pYV^+.

5.3.2 Fatty Acid Composition

The fatty acid compositions were determined of the two isogenic W22703 strains and the wild type strains 89.029 and 89.430. The major fatty acids were C16:0, C16:1, C18:1, cyclopropane-C17:0 and cyclopropane-C19:0. With all four bacterial strains, these five fatty acids represented 84-97% of the total fatty acid content (Figure 5.3).

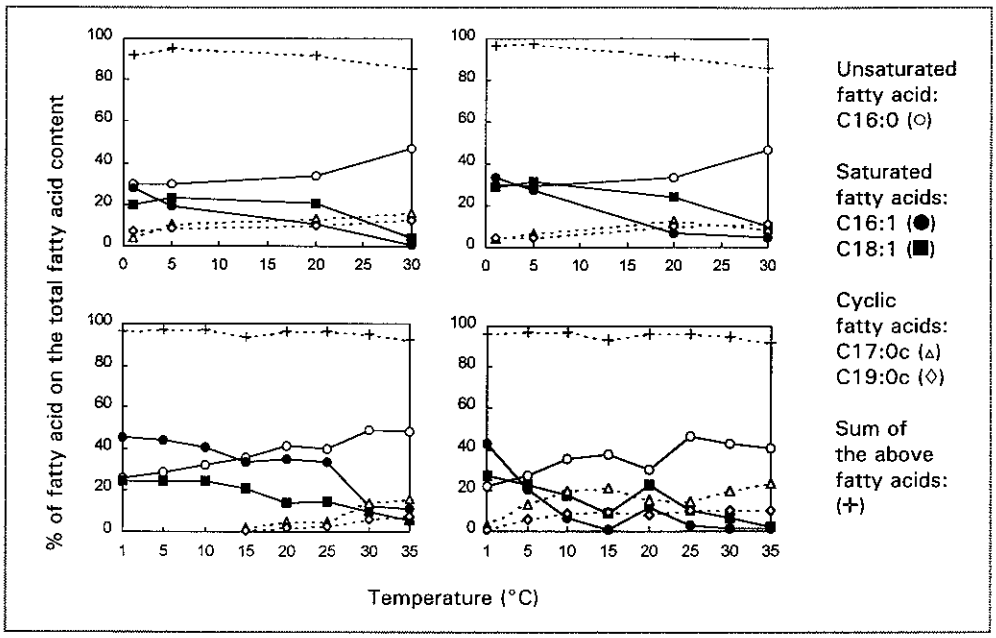


Figure 5.3 Fatty acid compositions of various *Y. enterocolitica* strains grown at various temperatures between 1 and 35°C. The graphs show the contribution of the main fatty acids, calculated as percentage of the total fatty acid content. Left side graphs concern pYV$^+$ strains, right side graphs are from pYV$^-$ strains. The upper graphs show strain W22703, and lower graphs are from strains 89.029 (left) and 89.430 (right).

At low growth temperatures, the unsaturated fatty acids predominated in all strains, ranging from 50-70% at 1°C to 40-65% at 10°C. At high temperatures (30-35°C), on the other hand, they comprised only 10-20% of the total fatty acid content. Conversely, the contribution of the saturated and cyclic fatty acids was low at 1-10°C (25-30% and 0-10%, respectively), and high at 30-35°C (50% and 20-25%, respectively). The fatty acid patterns of the two isogenic W22703 strains were quite similar to each other all over the temperature range (Figure 5.3: upper part). With the wild type strains, however, several marked differences were observed, including the absence of cyclic fatty acids at temperatures ≤15°C in strain 89.029 (pYV$^+$) which clearly contrasted with the situation in strain 89.430 (pYV$^-$) (Figure 5.3: lower part). In addition, strain 89.029 showed a high, and relatively constant C16:1 content (45-35%) over a broad temperature range (1 to 25°C), whereas the C16:1 content in strain 89.430 was high at 1°C only, and dropped rapidly with increase of the growth temperature.

When the U/S-ratio was calculated (i.e. the sum of the unsaturated fatty acids versus the sum of the saturated + cyclic fatty acids), it appeared that this U/S-ratio gradually decreased with increasing growth temperature, i.e. from 1.2-2.7 at 1°C to 0.1-0.3 at 30°C (Figure 5.4).

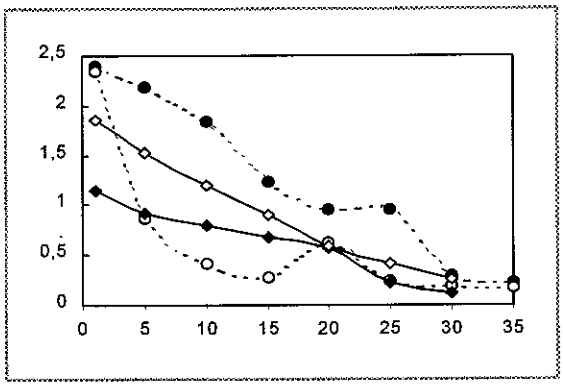


Figure 5.4
U/S-ratio in the fatty acid patterns of various *Y. entercolitica* strains grown at temperatures between 1 and 35°C. On the x-axis is the temperature (in °C); on the y-axis is the U/S ratio, calculated as the ratio of unsaturated versus the sum of saturated + cyclic fatty acids. Virulent, plasmid-harbouring strains were: W22703 /pYV$^+$(◆) and 89.029 (●). Non-virulent (plasmidless) strains were: W22703/pYV$^-$ (◇) and 89.430 (○).

5.3.3 Protein Analysis

Protein analysis of whole cells of strains 89.029 and W22703 pYV$^+$ showed that the plasmid-encoded Yad1 protein was present, as a 220 kDa band, at 25-35°C, but not at 20°C or below (Figure 5.5). As was to be expected, this band was not observed with the plasmidless strains 89.430 and W22703 pYV$^-$. Since we were mostly interested in specific bands at low temperatures, we carefully inspected the gels for differences between 1-5°C on the one hand and 10-35°C on the other hand. However, we only found minor differences between these growth temperatures. Weak bands of ± 30 kDa were observed at 1 and 5°C in W22703 pYV$^+$ and 89.029, while these bands were could not be detected in preparations of these strains grown at 20 and 30°C, nor in W22703 pYV$^-$ or in 89.430, at any temperature. Unfortunately, these results could not be reproduced when the analysis was repeated using gels with a high resolution in the low mass range (results not shown).
In the protein contents of cell envelope- or cytosol fractions, prepared from strains 89.029 and 89.430, no differences other than those concerning the 220 kDa YadA band could be detected (results not shown).
Finally, the concentrated cell free supernatants did not contain detectable amounts of protein.

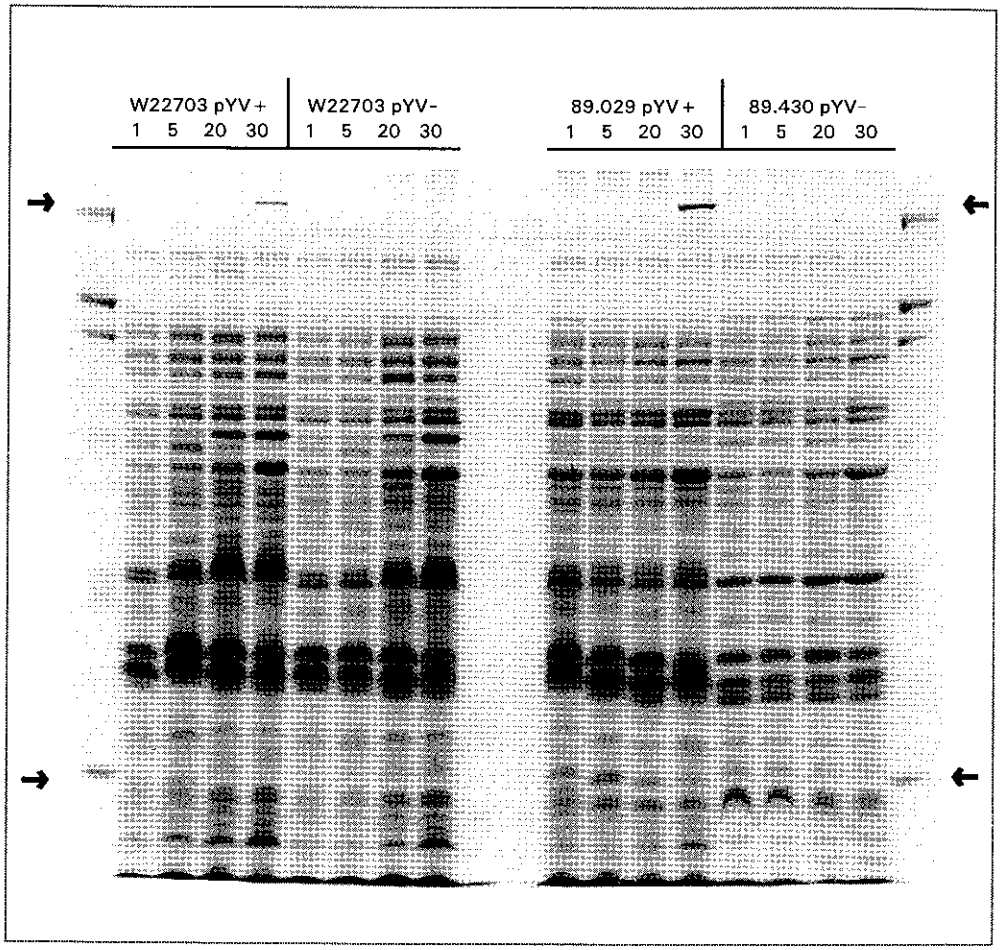


Figure 5.5 Protein patterns of virulent (plasmid harbouring) and non-virulent (plasmidless) strains of *Y. enterocolitica* at various temperatures.
Equal amounts of the total protein contents of strains grown at 1, 5, 20 or 30°C were separated on SDS-PAGE, and visualized by Coomassie Brilliant Blue staining. The strain numbers and genotypes, as well as the growth temperatures, are indicated above the lanes. The first and last lanes contained protein weight markers, showing bands at 200, 116, 97, 66, 45, and 31 kDa. Arrows point to the 220kDa YadA (Yop1) bands in the pYV^+ strains grown at 30°C, and to the unknown 30 kDa bands that are present only in the pYV- strains grown at 1 or 5°C.

5.3 DISCUSSION

Effects of pYV on Growth at Various Temperatures

Unlike most other members of the family of Enterobacteriaceae, *Y. enterocolitica* is capable to grow at refrigerator temperatures [Van Pee & Stragier 1979; Buckeridge *et al.* 1980; Kendall & Gilbert 1980]. Unfortunately, little is known about the regulation of this property, although it is known that the organism has several temperature-dependent, virulence plasmid-mediated properties, such as Yop-production and fibrillae formation [Lachica *et al.* 1984; Kapperud *et al.* 1987]. The massive production of Yops, at temperatures of 25°C and above, is attended by severe growth retardation. We speculated that (i) excessive production, if any, of a low-temperature-specific factor would be reflected in a similar growth retardation and (ii) factor(s) which allow the organism to grow at refrigerator temperatures might be detected by comparison of cellular compositions at high and low temperatures. To verify these assumptions, we first investigated the possible role of the virulence plasmid at different temperatures (1-35°C), using two isogenic reference strains and four wild type strains.

It was observed that the two plasmidless strains grew faster than the two plasmid-harbouring strains, not only at 30-35°C, but all over the temperature range. Although we could not differentiate between plasmid-born and serotypic effects in the wild-type strains, these results suggested that maintenance of the virulence plasmid is accompanied by some metabolic stress that retards the replication of the bacterium. Interestingly, the differences in growth rate of pYV^+ and pYV^- strains were more pronounced at high and low temperatures (i.e. at 25-35°C, and at 1-10°C) than at intermediate temperatures (15-20°C). The relatively slow growth of plasmid-harbouring strains at temperatures $\geq$ 30°C can be explained as the effect of metabolic stress due to both plasmid replication and the synthesis of YadA. Growth rate differences between pYV^+ and pYV^- strains at temperatures below 30°C, on the other hand, possibly only reflects stress due to plasmid replication, since no massive production of proteins is seen under these conditions. However, plasmid replication is not likely to account for more delay in growth at temperatures $\leq$10°C, than at 15-20°C. In theory, loss of the plasmid at temperatures of 15°C and above could account for unexpectedly diminished growth delay at 15-20°C, but this is not very likely, as no relevant plasmid loss was detected at any of the temperatures. Hence, it could be speculated that the greater differences that we observed between pYV^+ and pYV^- strains at temperatures $\leq$ 10°C than at 15-20°C reflect the production of a plasmid mediated, low-temperature-specific factor. However, such a plasmid-mediated factor is not likely to play a role in the organisms ability to grow at refrigerator temperatures, since both pYV^+ and pYV^- variants can grow under these conditions.

No Cold-Acclimation-Proteins Detected

Therefore, genome encoded factor(s) are more likely to be responsible for the capacity to growth in the cold. To find such factors, we analyzed the protein patterns of the strains grown

at various temperatures, but we were unable to detect clear differences (other than the 220 kDa YadA band) between isolates from different temperatures or between pYV^+ and pYV^- strains. When protein patterns of individual strains grown at different temperatures were compared, we found weak bands of ± 30 kDa in the pYV^+ strains when grown at 1 and 5°C only, but this observation could not be reproduced in gels with a high resolution in the low mass range. It was concluded that *Y. enterocolitica* does not produce vast amounts of low temperature specific proteins. Nevertheless, small amounts of low-temperature specific proteins might be present, but the detection of those apparently requires more specific or more sensitive techniques.

High Degree of Unsaturation in Short Chain Fatty Acids

The results of the analysis of the fatty acid patterns were in accordance with previous data [Abbas & Card 1980, Nagamachi *et al.* 1991]. Compared to those data, we found slight differences, i.e. the absence of C15:0/C17:0 and the presence of cyclopropane-C19:0 in our strains. The pYV^+ and pYV^- wild type strains differed substantially from each other in cyclic fatty acid contents at 1-15°C and, additionally, in C16:1 contents at 5-25°C. However, this is probably a serotypic, instead of a plasmid-born, effect, as these differences were not seen when comparing W22703 pYV^+ and pYV^-. It may therefore be concluded that thermal adaptation in *Y. enterocolitica* is not essentially influenced by the virulence plasmid.

When the various results with *Y. enterocolitica* are compared with those of *E. coli* [Marr & Ingraham 1962; Russell 1984], it might be speculated that these organisms use different strategies in thermal adaptation. The U/S-ratio of *E. coli* gradually increases from 1.5 to 3.4 (at 35°C and 10°C, respectively) and this effect that is almost entirely due to an increase in the C18:1 content. The U/S-ratio in *Y. enterocolitica*, on the other hand, is much lower all over the temperature range (0.2 at 30°C, 0.4-1.8 at 10°C, and 1.1-2.4 at 1°C), while its increase at temperature fall is mainly due to higher contents in C16:1 rather than in C18:1. This suggests that C16:1 plays an important role in maintaining membrane fluidity and permeability at low temperatures in *Y. enterocolitica*, or, in other words, that a high degree of unsaturation is not the only factor involved in the organism's adaptation to cold environments.

Conclusions

In conclusion, the results of this study suggest that an optimum degree of thermal adaptation in fatty acid composition allows *Y. enterocolitica* to grow at refrigerator temperatures. However, there is still no insight in the mechanism(s), or in the factor(s), which might regulate this process. There was a slight indication of a plasmid-encoded low-temperature specific protein factor but it implication could not be demonstrated. Molecular biological experiments are now in progress to trace the genetic origin of the suspected factors.

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6

Temperature & Genotype

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The psychrotrophic bacterium *Yersinia enterocolitica* requires expression of *pnp*, the gene for polynucleotide phosphorylase, for growth at low temperature (5°C)

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ABSTRACT

The psychrotrophic bacterium *Yersinia enterocolitica* is characterized by temperature-dependent adaptations. To investigate *Y. enterocolitica* genes involved in cold adaptation, a mutant restricted in its ability to grow at 5°C was isolated from a transposon mutant library. The transposon-insertion site in this psychrotrophy-defective (PD) mutant mapped sixteen base pairs upstream of an open reading frame whose predicted amino acid sequence showed 93% similarity with the *Escherichia coli* exoribonuclease polynucleotide phosphorylase (PNPase), encoded by *pnp*. Expression of this gene was blocked in the PD-mutant. However, introduction of a second copy of *pnp*, including 0.33 kbp sequences upstream of its coding region, into the chromosome of the PD-mutant restored *pnp* expression as well as the ability to grow at 5°C. Furthermore, expression of *pnp* appeared to be temperature-dependent: in the parental *Y. enterocolitica* strain, the levels of both *pnp*-mRNA and PNPase were 1.6-fold higher at 5°C compared to 30°C. A similarly enhanced level of PNPase at 5°C was observed in the merodiploid recombinant strain, which indicates that the 0.33 kbp region upstream of *pnp* harboured a cold-inducible promoter. A putative cold shock promoter motif (ATTGG) was observed in this region.

6.1 INTRODUCTION

Infection with *Yersinia enterocolitica* is an important and still increasing cause of gastro-enteritis in humans and is sometimes complicated by septicaemia or with the risk of subsequent reactive arthritis [Bottone 1977; Aho *et al.* 1981; Carniel & Mollaret 1990; Ostroff 1995]. *Y. enterocolitica* is considered to be mainly a food-borne pathogen and pork products are suspected to be the most likely source of infection [Schiemann 1989; Kapperud 1991; Nesbakken 1992]. In addition, reports of blood transfusion-associated *Y. enterocolitica* sepsis with a high mortality rate indicate a new mode of transmission for this organism [Wagner *et al.* 1994, Bottone 1997]. Advances in meat production, slaughter techniques, packaging and refrigeration allow industry and consumers to store foods for much longer periods, and technological break-throughs also permit longer storage of blood products. Under such circumstances, *Y. enterocolitica,* which is able to grow even at temperatures close to 0°C, can eventually grow out to clinically significant levels [Hanna *et al.* 1977; Kendall & Gilbert 1980; Brocklehurst & Lund 1990; CDC 1991]. In developing strategies to control its growth at low temperature, it is essential to understand which mechanisms are involved in cold adaptation of *Y. enterocolitica.*

Two major responses of bacteria to temperature down-shifts have been identified: alterations in the membrane lipid composition and changes in protein contents. The alterations in the lipid composition are manifested most commonly as changes in the relative proportions of the various fatty acid classes present and are supposed to provide a homeostatic mechanism for regulating membrane lipid fluidity [McElhaney 1976; Russell 1984]. The main cold-compensatory shifts are an increase in lipid unsaturation and a decrease in the average fatty acyl chain length, and both these alterations have been observed in mesophilic as well as in cold-tolerant organisms [Russell 1990], including *Y. enterocolitica* [Herbert 1986; Nagamachi *et al.* 1991; Goverde *et al.* 1994]. Nevertheless, psychrophilic and psychrotrophic bacteria, unlike mesophiles, seem to have developed the genetic ability to synthesize quantitatively or qualitatively more fluid lipids at low temperature [Gounot 1991]. This would explain how cold-tolerant micro-organisms can maintain the efficiencies of such essential functions as electron transport, ion pumping and nutrient uptake, but a direct relationship between membrane adaptations and the minimum temperature for growth has not been demonstrated.

The cold-induced changes in protein contents of bacteria are characterized by over-expression of certain subsets of proteins. Those which are specifically produced by psychrotrophs and psychrophiles during continuous growth at low temperatures have been called 'Cold Acclimation Proteins' (CAPs), whilst those which are transiently induced in response to rapid temperature downshocks are known as 'Cold Shock Proteins' (CSPs). The cold shock response was first observed in the mesophile *E. coli* [Jones *et al.* 1987], but has now been documented for many other micro-organisms, including heat- and cold-tolerant species [Jones & Inouye 1994]. Many of the CSPs produced by *E. coli* have now been identified and these were shown to be

involved in such diverse cellular processes as termination of transcription, initiation of translation, mRNA-degradation, pyruvate-decarboxylation, or DNA-supercoiling [Jones *et al.* 1987, 1992, 1996; Jones & Inouye 1994, 1996]. Strong indications exist that the major *E. coli* cold shock protein, CS7.4 or CspA, is involved in transcriptional activation of genes from other cold shock proteins by specific binding to 5'-CCAAT-3' or 5'-ATTGG-3' motifs in the promoter regions of these genes [LaTeana *et al.* 1991; Qoronfleh *et al.* 1992; Jones & Inouye 1994]. Comparative studies have shown that there is considerable, but not complete, overlap between the products of cold acclimation and the cold shock response [Roberts & Inniss 1992, Whyte & Inniss 1992; Gumley & Inniss 1996; Panoff *et al.* 1997]. CAPs which are highly homologous to the *E. coli* CspA have been found in various psychrophilic or psychrotrophic species, including *Pseudomonas fragi* [Hébraud *et al.* 1994], *Bacillus cereus* [Mayr *et al.* 1996] and *Arthrobacter globiformis* [Berger *et al.* 1996] and it was therefore suggested that the continuous over-expression of these CspA-like proteins might contribute to the ability to grow at low temperature [Berger *et al.* 1997]. It has also been suggested that mesophilic bacteria fail to restore harmonious growth at low temperatures because their cold shock response is only a part of the total response of cold-adapted bacteria [Berger *et al.* 1996], but comparative studies addressing the CSP-profiles of wild-type and cold-sensitive mutant strains of psychrotrophic bacteria show that additional factors must be involved [Whyte & Inniss 1992; Bayles *et al.* 1996].
In this chapter, the generation and isolation of transposon-induced mutants of *Y. enterocolitica* which are severely hampered in growth at 5°C is described, as well as the subsequent identification of *pnp* as the affected gene and the identification of its product, polynucleotide phosphorylase, as a cold acclimation protein. In addition, complementation- and expression experiments provide evidence consistent with the ATTGG motif playing a role in cold enhanced expression of the *pnp* gene in *Y. enterocolitica*.

6.2 MATERIALS & METHODS

6.2.1 Bacteria, Plasmids, Growth Conditions

Bacterial strains and plasmids and relevant characteristics, are listed in Table 6.1. *E. coli* strains were cultured in NZ-broth or on NZ-agar (Bio101, Vista CA, USA) at 37°C. *Y. enterocolitica* strains were cultured at 30°C or 5°C in NZ-broth, tryptic soy broth (Difco, Detroit MI, USA), or RPMI-1640 broth (Gibco BRL, Inchinnan, Scotland) or on NZ-agar, tryptic soy agar or plate count agar (PCA, Difco). When required, the media were supplemented with the following antibiotics: ampicillin (100 µg/ml); kanamycin (100 µg/ml); nalidixic acid (35 µg/ml); or streptomycin (100 µg/ml). Strains were frozen in 15% glycerol at -70°C for permanent collection.

Table 6.1 Bacterial strains and plasmids

STRAINS	RELEVANT CHARACTERISTICS	REFERENCES
Y. enterocolitica		
W22703	Naladixic acid^R, pYV$^-$	*13*
YM203/204 205/206/208	Psychrotrophy-Defective (PD) transposon mutants of strain W22703	*this work*
YM207	Semi-PD transposon mutant of W22703	*this work*
YM222	Non-PD transposon mutant of W22703	*this work*
YM607	Merodiploid strain derived from YM205 with an integrated copy of pRG547	*this work*
YM588	Merodiploid strain derived from W22703, with an integrated copy of pRG547	*this work*
E. coli		
S17.1 λpir$^+$	Streptomycin^R; host for pKNG113	*60*
SM10 λpir$^+$	Kanamycin^R; used as a host for pRG547	*60*
SY327 λpir$^+$	Nalidixic-acid^R; used as a host for pRG547	*42*
DH5α	*rec*A1, *lacZ*$^-$, used as a host for pRG5, pRG112, pRG315 and pRG514	*Gibco BRL*
EcRG558	SM10 λpir$^+$, harbouring pRG547	*this work*
PLASMIDS		
pKNG113	Suicide vector, derivative of Tn5; *oriR6K, oriT*; kanamycin^R	*Dr. Kaniga (pers.comm.)*
pKNG101	Suicide vector, *oriR6K, oriT, mobRK2*; streptomycin^R, sucrose^S	*34*
pUC21BM$^+$	Cloning vector; ampicillin^R	*Boehringer*
pRG5	pUC21BM$^+$ with 3.3 kbp *Eco*RI-*Hind*III chromosomal fragment from YM205, containing the transposon insertion site and adjacent *yhbB*, *yhbA* and *rpsO* genes; kanamycin^R	*this work*
pRG112	pUC21BM$^+$ with 3.0 kbp *Eco*RI-*Cla*I chromosomal fragment from W22703, containing *yhbB*, *yhbA*, *rpsO* and the 5'-terminus of *pnp*	*this work*
pRG315	pUC21BM$^+$ with 3.2 kbp inverse-PCR product of *Eco*RI fragment of W22703, containing *pnp* and the 3'-terminus of *rpsO*; ampicillin^R	*this work*
pRG514	pUC21BM$^+$ with *rpsO'* and *pnp* from W22703, subcloned from pRG315	*this work*
pRG547	pKNG101 with *rpsO'* and *pnp* from W22703, subcloned from pRG514	*this work*

6.2.2 General Recombinant DNA Methods

Chromosomal DNA isolation, cloning of DNA fragments, transformation, plasmid isolation and agarose gel electrophoresis were performed as described elsewhere [Ausubel *et al.* 1987]. Restriction enzymes were used according to the instructions provided by the manufacturer (Boehringer Mannheim, Germany or Gibco-BRL/Life Technologies, Gaithersburg, MD, USA).

Transformations were performed by electroporation at settings: 2.5 kV, 200 Ω, 25 μF, using 0.2 mm cuvettes in a Gene Pulser apparatus (BioRad, Hercules, CA, USA). For Southern blotting, chromosomal DNA was digested to completion with restriction enzymes, separated by agarose gel electrophoresis, denatured, and transferred to Hybond N^+ membranes (Amersham International, Amersham, UK) by vacuum blotting (Milliblot V system, Millipore). DNA fragments were purified from agarose gel using GeneClean (Bio 101) or QiaEx (Qiagen Inc, Chatsworth, CA, USA). Probes were labelled and hybridizations were performed using the enhanced chemiluminescence (ECL) gene detection system (Amersham). Primers used for PCR- and sequencing reactions are listed in Table 6.2.

Table 6.2

DNA sequences of the primers used for PCR and sequencing. The sequences are given from 5' to 3'. The genes harbouring the primer sequences are indicated. The position numbers correspond with the numbering of the respective nucleotides in the sequence that was submitted to EMBL (accession number Y10692).

Primers	Sequence	Gene	Position
Forward			
1F	CATATTGTCGTTAGAACGCG	*tnp*	
6F	CGTAAGCTGCTGGACTACCT	*rpsO*	1701-1720
4F	AGCTACTGGGCTATCTGGAC		
8F	CCGCAGTATTCGTTACTGTT	*pnp*	2151-2170
9F	CGTGTTGGTTTTATCAATGA	*pnp*	2496-2515
10F	GGTAGCGTTGAGAAAGACGT	*pnp*	2931-2950
12F	GAAATCACCGAGTCTAACGG	*pnp*	3327-3346
13F	GGATGTGATCGGTAAAGGCG	*pnp*	3737-3756
Reverse			
2R	CTTTCTACGTGTTCCGCTTC	*kan*	
5R	GATGTAACGCACTGAGAAGC		
7R	GACGTGAAGTCAGTGTTTCG	*pnp*	2319-2300
11R	CTGCATTTCCTGTGAAACAC	*yhbB*	39-20
14R	TTTAACACAAGAGCATCCTG		4233-4214
15R	CTCTTCGATACGACGGATAG	*pnp*	3881-3862
16R	GGCTACCGGCTACTTTAAAG	*pnp*	3534-3515
17R	CATCAATATTTTGTGCGTCA	*pnp*	3138-3119
18R	CAACCAGAGCGTTGATATTT	*pnp*	2709-2690

PCR reactions were performed in a final reaction volume of 25 μl, using a type 480 DNA thermocycler (Perkin Elmer, Foster City, CA, USA). The reaction mixtures contained 0.5 U of Taq DNA polymerase (Perkin Elmer), 10 pmol each of two primers, standard amounts of amplification reagents {200 μM of each deoxyribonucleotide triphosphate (dNTP); 50 mM Tris-HCl, pH 9.0; 50 mM KCl; 1.5 mM $MgCl_2$; 0.01% gelatin; 0.1% Triton X-100} and 5 μl of a solution containing 1 pg to 10 ng of template DNA. Template solutions were either cleared cellular lysates, prepared by resuspending a loopfull of colony material in 100 μl 0.9% NaCl, heating for 20 min at 95 °C and pelleting by centrifugation, or appropriate dilutions of isolated

DNA. PCR involved a pre-heating for 3 min at 95°C, 30 cycles of consecutive denaturation (1 min at 95°C), primer annealing (1 min at 55°C) and chain extension (3 min at 72°C), and an additional extension step of 7 min at 72°C. DNA sequencing reactions were performed using 'Ready-Reaction Dye-primer' and '-Dye Terminator' sequencing kits, according to the instructions provided by the manufacturer (Applied Biosystems, CA, USA). Sequences were determined using an Applied Biosystems 373A automated DNA sequencer. The sequence was submitted to the EMBL/Genbank Data Library (accession number Y10692). DNA and protein sequences were analyzed, and aligned with *E. coli* (accession number J02638) and *P. luminescens* (accession number X76069) sequences present in the EMBL/GenBank databases, using the BLAST and FASTA programs [Altschul *et al.* 1990; Pearson & Lipman 1988].

6.2.3 Induction and Isolation of PD-mutants

Library construction

A transposon-insertion mutant library of *Y. enterocolitica* was constructed by plate mating with an *E. coli* strain harbouring the transposon on a suicide plasmid, essentially as described by [Cornelis *et al.* 1991]. Briefly, equal volumes of log-phase cultures of the nalidixic-acid resistant strain *Y. enterocolitica* W22703 and the *E. coli* strain S17.1 harbouring pKNG113, which carries a kanamycin resistance gene on a derivative of Tn5, were mixed on a TSA plate and incubated ON at 32°C to enable conjugation. Subsequently, the plate culture was suspended in 0.9% NaCl and kanamycin/nalidixic-acid resistant recombinants were amplified on selective agar plates. Approximately 40,000 kanamycin/nalidixic-acid resistant colonies were obtained, which were pooled and resuspended in freezing medium {50% glycerol; 2.5 mM $MgSO_4$; 5 mM Tris-HCl, pH=7.4} and stored at -70°C for permanent collection.

Enrichment and isolation

Mutants which were restricted in their growth at low temperature were isolated from the transposon-mutant library by negative enrichment at the non-permissive temperature, essentially as described by [Hooke *et al.* 1991]. Briefly, the mutant library was cultured in RPMI-1640 at 5°C until early log phase after which D-cycloserine was added to give a final concentration of 1 mg/ml. Incubation at 5°C was continued for 146 h. Samples were taken at regular intervals and the amount of surviving cells was determined by viable colony counts at 30°C. Several hundred colonies, obtained at different time points, were replica plated, incubated at 30°C and 5°C, and those unable to produce colonies at 5°C within 20 days identified and frozen for permanent collection.

Putative psychrotrophy-defective (PD)-mutants obtained with this approach were subjected to further characterization by monitoring growth rates in broth and/or plate cultures at 30°C and 5°C. Growth on plates was recorded by measuring mean colony-diameters at regular intervals as previously described [Goverde *et al.* 1994] and growth profiles in liquid medium were

determined either spectrophotometrically or by viable count determinations. Mutants were classified as PD, semi-PD or non-PD according to their respective growth profiles.

6.2.4 Identification, Cloning, and Analysis of the Affected DNA Region

The DNA regions which harboured the transposon in the PD-mutants, and the corresponding region in the parental strain, were identified by Southern analysis, using various probes. Relevant fragments were cloned into pUC21BM$^+$, and analyzed by sequencing. A map of the region is given in Figure 6.1. The positions of the probes and primers used for identification and (sub)cloning of the relevant fragments are indicated, as well as the cloned fragments and corresponding constructs.The constructs are listed in Table 6.1.

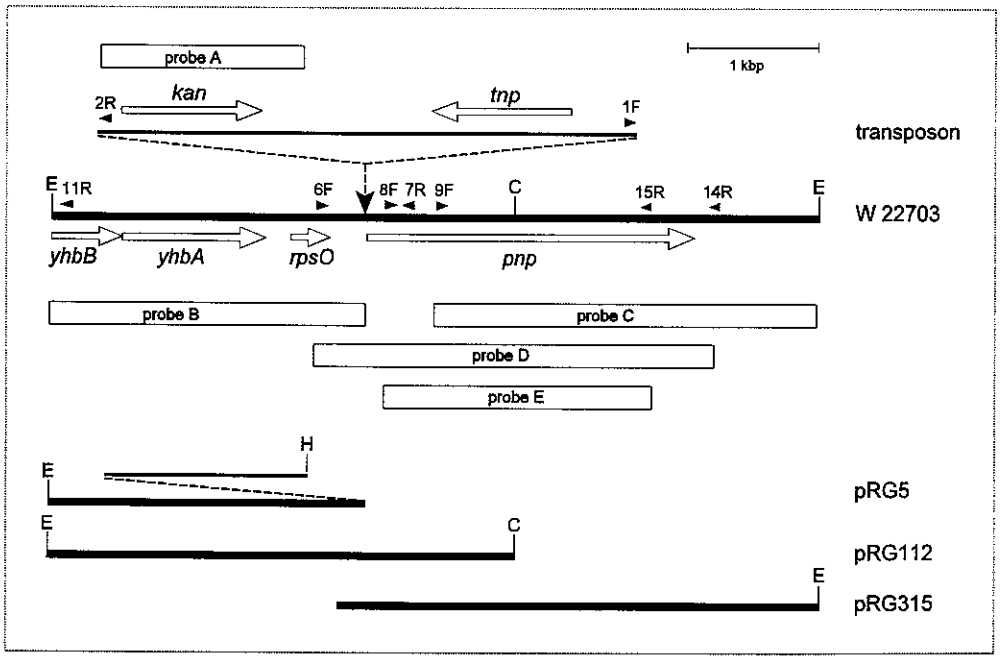

Figure 6.1
Genetic and physical map of the *Y. enterocolitica* DNA region containing the transposon insertion which affected psychrotrophic growth. The heavy lines representfragments of *Y. enterocolitica* W22703.The upper part shows a 4.9 region in W22703. The transposon is shown as a thin line above the *Yersinia* fragment, and its insertion site in the PD-mutant YM205 is indicated (▾). The position and orientation of genes in this region are indicated by arrows, and those of relevant primers by arrowheads (▸, ◂). Probes used in this study are shown as open boxes. Cloned DNA-fragments, and the numbers given to the corresponding plasmid constructs, are indicated at the bottom of the figure. The restriction sites indicated are: C = *Cla*I, E = *Eco*RI, H = *Hin*dIII.

Probe A was obtained by PCR-amplification of the transposon with primers 1F/2R, using pKNG113 as a template, and was used used for identification of the insertion region in the PD-mutants. A 3.3 kbp *Eco*RI-*Hin*dIII fragment hybridizing to this probe was cloned into the *Eco*RI-*Hin*dIII digested vector, resulting in the plasmid pRG5. *Eco*RI-*Cla*I fragments from W22703, ranging in size from 2.8 to 3.6 kbp, were cloned into the *Eco*RI-*Cla*I digested vector, and probe B (derived from pRG5) was used to identify pRG112 as a clone that contained the corresponding wild type region. The complete gene of interest was cloned by means of inverse PCR [Ochman *et al.* 1990], resulting in plasmid pRG315 (depicted in Figure 6.2, upper part). Briefly, a 4.9 kbp *Eco*RI fragment from W22703 was self-ligated and amplified using primers 6F and 11R. The 3.2 kbp product was, after treatment with T4- DNA polymerase and *Eco*RI, cloned into the *Eco*RI-*Eco*RV digested vector. Probe C, which was obtained by PCR from the self-ligated 4.9 kbp *Eco*RI fragment using primers 9F/11R, was used to identify pRG315 as a clone that contained the fragment of interest.

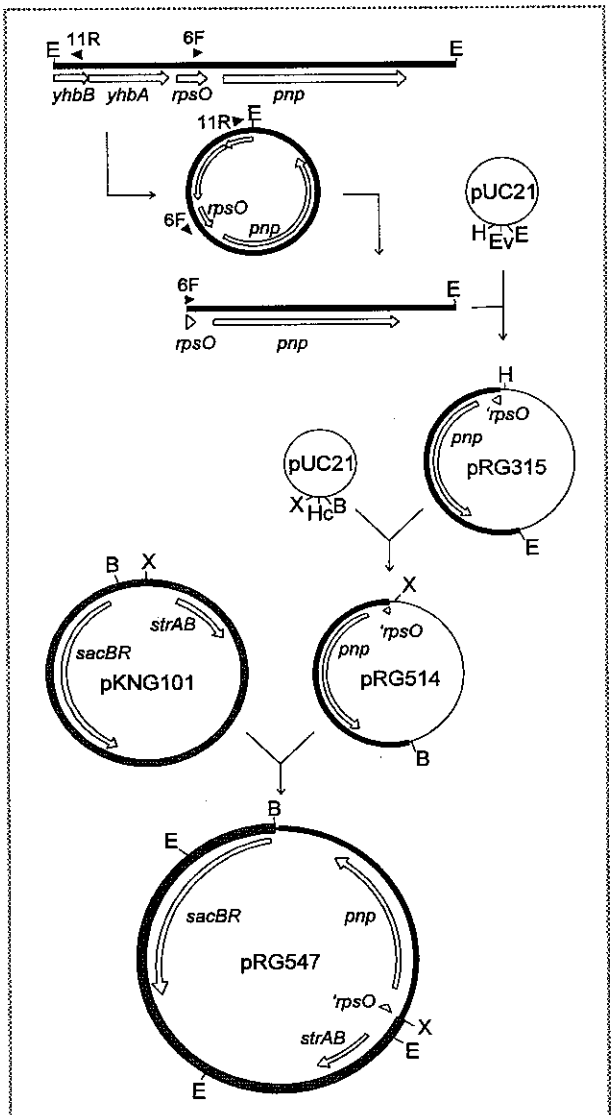

Figure 6. 2
Construction of plasmids containing the *pnp* gene of *Y. enterocolitica* W22703, including its presumed transcription and translation signals. *Yersinia* DNA is shown by heavy lines. The cloning vector is depicted by thin lines and the suicide vector in shown in grey. The position and orientation of relevant genes and primers are indicated by arrows and by arrowheads, respectively.
Relevant restriction sites are:
B = *Bam*HI, E = *Eco*RI, Ev = *Eco*RV, H = *Hin*dIII, Hc = *Hin*cII, and X = *Xba*I.

6.2.5 Complementation of the PD-mutation, and Construction of Mero-diploid Strains

A suicide construct which contained the wild type *pnp* gene, including its flanking regions (pRG547), was used to study whether the PD-mutation could be complemented with *pnp*, and to study a possible gene-dose effect in the wild type parental strain. The strategy for the construction of pRG547 is shown in Figure 6.2 (lower part). Briefly, a 3.2 kbp *Eco*RI/*Hin*dIII fragment of pRG315 was treated with T4 polymerase and subcloned into the *Hin*cII-digested vector pUC21BM$^+$, resulting in pRG514. A 3.2 kbp *Bam*HI/*Xba*I fragment excised from pRG514 was inserted into the *Bam*H1/*Xba*I-digested vector pKNG101, resulting in pRG547, which was harboured in *E. coli* SM10. This host strain (Table 6.1: EcRG558) was conjugated to one of the PD-mutants, essentially as described by [Kaniga *et al.* 1991], to introduce pRG547 into the chromosome of the mutant via homologous recombination. Exconjugants with the desired insertion were identified by PCR, using primer pairs 1F/7R and 6F/7R, and by Southern analysis, using probe A and probe D (amplified from pRG315, with primers 6F/14R). To investigate the occurrence of a gene-dose effect, strains harbouring two intact copies of *pnp* were constructed by insertion of pRG547 into the chromosome of W22703, essentially as described above.

6.2.6 Immunoblotting

Broth cultures of the strains of interest, grown at either 30°C or 5°C to an OD_{600nm} of approximately 0.7, were centrifuged for 1 min (12000 g) and the cell pellets were washed twice in PBS. When necessary, pellets were stored until use at -20°C. The pellet was resuspended in sonication buffer {10 mM Tris-HCl, pH=7.5; 5 mM $MgCl_2$} and cells were disrupted on ice by four sonic bursts of 10 sec (amplitude 10μ), interrupted by 20 sec pause, using an ultrasonic desintegrator (MSE Scientific Instruments, Crawley, Sussex, England). Debris and unbroken cells were removed by centrifugation (12000 g) for 10 min at 4°C. Protein concentrations in the supernatants were determined using a BCA Protein Assay (Pierce Chemical Co., Rockford, IL, USA). Equal amounts of total cellular protein were separated by SDS-PAGE (10%) and transferred to nitrocellulose membranes (Hybond-C-extra, Amersham International, Amersham, UK). Polynucleotide phosphorylase was assayed by immunostaining using rabbit anti-PNPase (kindly donated by Dr. Portier), anti-rabbit alkaline-phosphatase (Boehringer Mannheim, Germany) and NBT/BCIP (Sigma Chemical Co., St.Louis, MO, USA). Quantification of the intensity of visible bands was accomplished using a GS-700 Imaging Densitometer (BioRad, Hercules, CA, USA).

6.2.7 RNA-spot-blot Analysis

Total RNA was isolated from broth cultures of the wild-type strain and the PD-mutant YM205

which had been incubated ON at 30°C or 5°C, using an RNEasy total RNA kit (Qiagen, USA) according to the manufacturer's instructions. To ensure complete removal of residual DNA, the RNA was treated with RNase-free DNase I (Promega, Madison, WI, USA). The integrity of the RNA was analyzed by gel-electrophoresis and the concentrations were determined spectrophotometrically. Samples were adjusted to equal concentrations by dilution with sterile water and amounts of 1 µg, 500, 250, 125, 62.5, 31, 15.6 and 7.7 ng of RNA were spotted onto positively charged nylon membrane (Boehringer Mannheim, Germany) using a spot blot apparatus (Biorad, USA). Duplicate membranes were hybridized with a 1.7 kbp PCR fragment of the *Y. enterocolitica pnp* gene, obtained with primers 8F and 15R (Figure 6.1, probe E), or with a 1.4 kbp PCR fragment derived from the 16S-rRNA gene of *Y. enterocolitica*, to check whether equal amounts of RNA of the four samples were spotted. Probes were labelled with [α^{32}P]-dATP using a 'Prime-it II random primer labelling' kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. Hybridizations were performed overnight at 68°C in hybridization solution containing {1% sodium dodecyl sulphate (SDS); 0.1% sodium lauryl sarcosinate; 1% Blocking Reagent (Boehringer Mannheim, Germany); 6x SSPE (SSPE: 180 mM NaCl; 10 mM sodium phosphate, pH 7.5; 1 mM EDTA) and 50 µl of the labelled probe}. Washings were performed at 68°C (3x 15 min) in {0.5% SDS; 0.1x SSPE}. The signals were quantified using a Phosphorimager (Molecular Dynamics, Sunnyvale, Ca, USA).

6.3 RESULTS

6.3.1 Isolation and Characterization of PD-mutants

A library of approximately 40,000 independent transposon insertion mutants of W22703 was obtained (data not shown). Twenty-five putative PD-mutants, which were survivors of the negative enrichment treatment with the cell wall antibiotic D-cycloserine, were isolated. The growth profiles of these strains at 30°C and 5°C were compared to those of the parental strain and it appeared that five of them were 'psychrotrophy-defective' (PD) since they (i) failed to produce colonies on agar within 50 days of incubation at 5°C and (ii) showed extremely delayed growth in broth cultures at 5°C, while they could not be distinguished from the parental strain at 30°C (Figure 6.3, and Table 6.1: YM203, YM204, YM205, YM206 and YM208). In addition, one strain showed moderate growth retardation, compared to the parental strain, at both 30°C and 5°C and this was designated semi-PD (Figure 6.3, Table 6.1: YM207). The remaining isolates could not be differentiated from the parental strain with respect to their growth rates at both 30° and 5°C (data not shown); these were considered not to be altered in their psychrotrophic phenotype and were therefore designated non-PD.

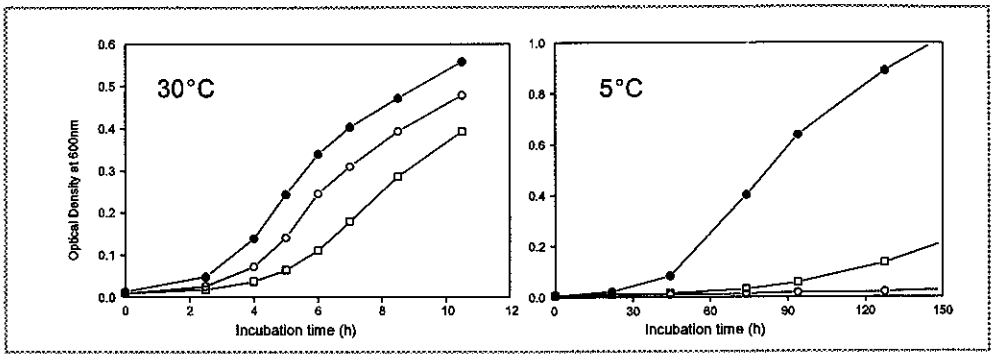

Figure 6.3 Growth profiles of *Y. enterocolitica* W22703 (●) and its mutant derivatives YM205(○)and YM207(□), at 30 and 5°C. The growth profiles of YM203, YM204, YM206, and YM208 were identical to those of YM205.

6.3.2 Analysis of the Affected DNA Region

To determine whether the distinct growth phenotypes of the mutants could be attributed to transposon insertions at different sites, we characterized the DNA regions harbouring the transposon. Southern blot analysis revealed a single 4.2 kbp *Eco*47III band in all five PD-strains, indicating that a single transposon insertion had occurred in corresponding regions of these mutants (Figure 6.4, lanes 1, 2, 4, 5 and 15).

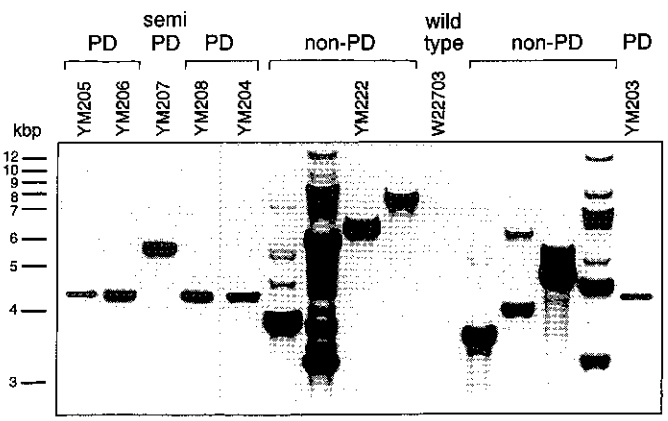

Figure 6.4
Southern blot analysis of *Y. enterocolitica* W22703 and several transposon mutant derivatives. Chromosomal DNA was digested with *Eco*47III and hybridized with a transposon-derived probe (Figure 6.1: probe A). The strain numbers and growth-phenotypes (PD, semi-PD and non-PD, as described in the text) are indicated at the top of the lanes. Numbers at the left side indicate sizes of standard DNA fragments.

In the semi-PD mutant, a single 5.1 kbp band hybridized to the probe (Figure 6.4, lane 3), which indicated that an insertion had occurred at a different site compared to the PD-mutants.

When a number of non-PD mutants was analyzed by Southern blotting, much more heterogeneity was observed in the number and sizes of the hybridizing fragments and none of these mutants contained a 4.2 kbp *Eco*47III band which hybridized with probe A (Figure 6.4, lanes 6-9 and 11-14).

We subsequently determined which gene was affected by the transposon insertion in the PD-mutant YM205. A 3.3 kbp *Eco*RI/*Hin*dIII DNA fragment that hybridized with probe A was cloned, resulting in plasmid pRG5 (Figure 6.1), and sequenced. Three successive open reading frames (ORFs) were observed in the *Yersinia* DNA. A data base search with the putative products revealed significant sequence identity with the *E. coli* proteins P15B (or RbfA), P35 and S15 [Takata *et al.* 1984; Sands *et al.* 1988; Dammel & Noller 1995]. P15B, P35 and S15 are encoded by, respectively, *yhbB*, *yhbA* and *rpsO* genes in *E. coli*, and we have given the same designation to the corresponding genes found in *Y. enterocolitica* (Figure 6.1). The transposon was not inserted in one of the three genes identified but appeared to be located some 250 bp downstream of the 3'-terminus of *rpsO*. This suggested that the insertion affected a gene which is located downstream of *rpsO*. To characterize this gene, DNA fragments from the wild-type strain containing sequences downstream of *rpsO* were identified by Southern analysis using probe B. Partly overlapping fragments of 3.0 kbp and 3.2 kbp were cloned, resulting in the plasmids pRG112 and pRG315, and sequenced. A large ORF was observed downstream of *rpsO*; and its predicted amino acid sequence revealed 86% sequence identity and 93% similarity with the *E. coli* exoribonuclease polynucleotide phosphorylase (PNPase), encoded by the *pnp* gene [Régnier *et al.* 1987]. Comparison of the affected region in YM205 with the corresponding region in the parental strain indicated that the transposon insertion had resulted in the duplication of nine base pairs of *Yersinia* DNA, whilst the transposon insertion site was located sixteen base pairs (including the duplicated sequence) upstream of the putative translation start codon of the *pnp* gene (Figure 6.5).

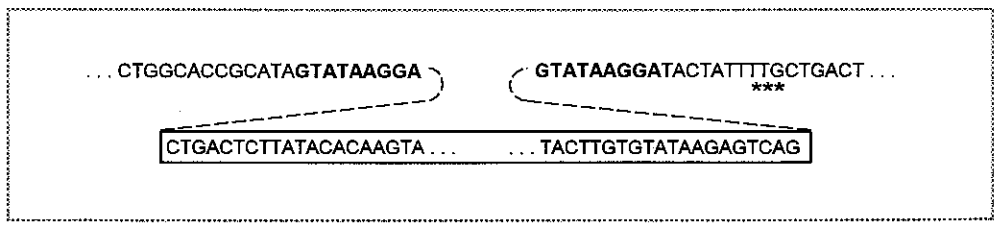

Figure 6.5 Genetic map showing the left and right junctions between *Y. enterocolitica* DNA and the transposon in the PD-mutants. The sequence is given from 5' to 3'. The upper row shows the *Y. enterocolitica* DNA. The first translated codon of *pnp* (TTG) is indicated by asterisks.The inserted transposon is depicted as a box. In this, only the inverted repeats are written in full; the sequence in between these extremities is depicted by dots.

The transposon insertion sites in the remaining PD-mutants were determined by PCR amplification of the DNA regions containing the presumed left and right junctions between *Yersinia* and transposon DNA, using primers deduced from *tnp*, *pnp* and *rpsO* (Table 6.2: primers sets 6F/2R and 1F/7R), and subsequent sequencing. PCR-products of identical size were obtained with all five PD-mutants, and sequence analysis revealed that the transposon insertion site in YM203, YM204, YM206 and YM208 was identical to that found in YM205. Using the same sets of primers, no PCR products were obtained with the semi-PD mutant, supporting the assumption that the transposon was inserted in a different region compared to the PD-mutants.

6.3.3 *PNP*-expression in the Wild Type strain and in PD-mutants

Since the transposon was inserted just upstream of the *pnp* gene in all five PD-mutants, it seemed likely that this insertion affected the expression of *pnp*. To investigate this assumption, PNPase levels in wild-type and mutant strains were determined semi-quantitatively by immunoblotting, using anti-serum against PNPase (Figure 6.6), and densitometric scanning of the signals.

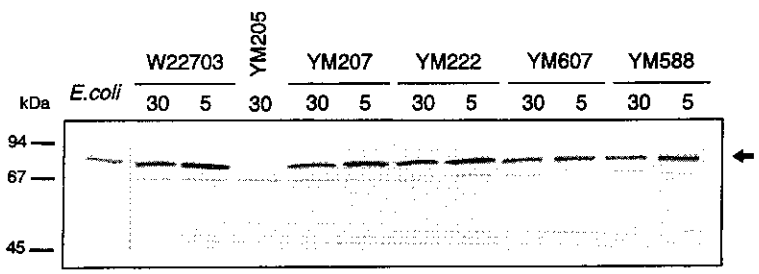

Figure 6. 6
Effect of growth temperature on the levels of PNPase in Y. enterocolitica, strain W22703 and five derivatives obtained in this study, i.e. three different transposon-insertion mutants and two merodiploid strains. Equal amounts (10μg) of total cellular protein, extracted from strains grown to midlog phase at 30°C or at 5°C, were analyzed on a Western blot using anti-PNPase polyclonal antiserum. Strain numbers and growth temperatures are indicated at the top of each lane. Derivative strains analyzed were either transposon insertion mutants of W22703 with varying growth phenotypes (as described in the text): YM205 (psychrotrophy-defective, PD), YM207 (semi-PD) and YM222 (non-PD), or merodiploid strains harbouring a second copy of *pnp*: YM588 (derived from W22703) and YM607 (derived from YM205). The first lane contained 2 μg purified *E. coli* PNPase (Sigma). Numbers at the left side indicate sizes of standard proteins in kilodalton. The position of PNPase is marked by an arrow.

The amounts of PNPase in the semi-PD mutant YM207 (lanes 5 and 6) and in a randomly chosen non-PD mutant (Table 6.1: YM222) (lanes 6 and 8), appeared to be approximately equal to that found in the wild-type strain (lanes 2 and 3), but PNPase could not be detected in the PD-mutant YM205 (lane 4), nor in the remaining PD-mutants (data not shown). Furthermore, the levels of PNPase in YM207, YM222 and the parental strain were 1.3 to 1.6-fold higher when the cells were grown at 5°C instead of 30°C (Table 6.3).

Table 6.3 Relative levels of PNPase and *pnp* mRNA in *Y. enterocolitica* W22703, three different transposon-mutants and two mero-diploid derivatives, at 30°C and 5°C.

STRAIN	PHENOTYPE	GENOTYPE	PNPase[1] 30°C	PNPase[1] 5°C	*pnp* mRNA[2] 30°C	*pnp* mRNA[2] 5°C
W22703	psychrotroph	*pnp* wild-type	1.0	1.6	1.00	1.59
YM205	PD[3]	*pnp* transposon insertion mutant	0.0	nm[4]	0.06	0.03
YM207	semi-PD	transposon mutant, not in *pnp*	0.9	1.3	nm	nm
YM222	non-PD	transposon mutant, not in *pnp*	1.0	1.5	nm	nm
YM607	psychrotroph	as YM205 + second copy of *pnp*	0.9	1.3	nm	nm
YM588	psychrotroph	as W22703 + second copy of *pnp*	0.9	1.5	nm	nm

[1] Total cellular proteins, extracted from cells grown at 30°C or 5°C, were PNPase-specific immunostained on Western blots, and the obtained signals were quantified by densitometry. The signal obtained with W22703 grown at 30°C was taken as a benchmark and the corresponding PNPase level was arbitrarily set at 1. The relative PNPase levels at 5°C and in the remaining strains were calculated from the corresponding densitometric signals. The figures are averages from the results of at least three independent experiments.

[2] Total mRNA, extracted from cells incubated ON at 30° or 5°C, was hybridized with a *pnp* specific DNA-probe and the obtained signals were quantified with a Phosphorimager. The signal obtained with W22703 incubated at 30°C was taken as a benchmark and the corresponding level of *pnp* mRNA was arbitrarily set at 1. The relative levels of *pnp* mRNA in W22703 at 5°C, and in YM205 were calculated from the corresponding signals.

[3] PD = psychrotrophy-defective

[4] nm = not measured

To investigate whether this temperature-dependent expression could be attributed to differences at the transcriptional level, we also determined *pnp* mRNA levels in the wild-type strain and in the PD-mutant YM205, and the results are summatrzed in Table 6.3. The levels of *pnp* mRNA in the wild-type strain were found to be 1.59-fold (± 0.02) higher in cells grown at 5°C compared to 30°C, whereas the amounts of *pnp* mRNA in the PD-mutant, either grown at 30°C or incubated overnight at 5°C, were negligible (Table 6.3). These results suggested that (i) *Y. enterocolitica* requires PNPase for growth at 5°C and (ii) expression of the *pnp* gene is, at least partly, under control of a cold-inducible promoter.

6.3.4 Complementation of the PD-mutation

In view of its proximity to the 5'-end of *pnp*, it seemed likely that the transposon insertion blocked transcription of *pnp* in the PD-mutants, and the observed absence of *pnp* mRNA in YM205 supported this assumption. In *E. coli*, transcription of *pnp* is mainly initiated downstream of *rpsO* [Portier *et al.* 1987] and the same was observed in *P. luminescens* [Clarke & Dowds 1994]. To determine whether this was also the case in *Y. enterocolitica*, the plasmid pRG315, which contained the cloned *pnp* gene and 330 base pairs upstream of the *pnp* translation startpoint, was introduced into the PD-mutant YM205. The upstream sequences comprised the 3'-end of *rpsO*, and the *rpsO-pnp* intercistronic region. Growth at 5 °C appeared to be restored in transformants of YM205 which harboured pRG315, although wild-type growth rates were not attained (Figure 6.7). These results indicated that the *pnp* promoter is located in the 330 base pairs DNA region directly upstream of the coding region of *pnp*.

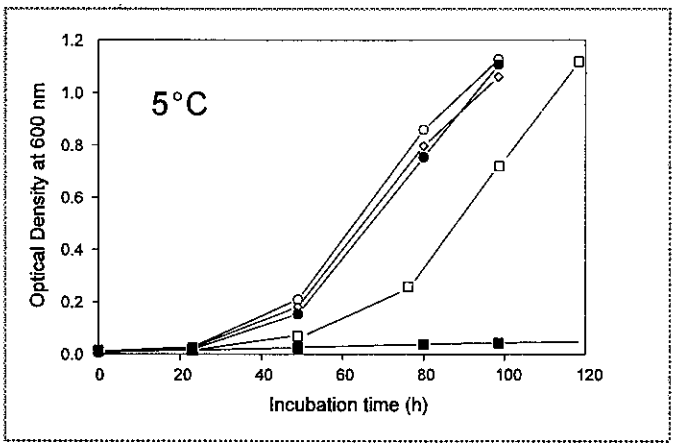

Figure 6.7
Growth profiles at 5°C of *Y. enterocolitica* W22703 (●) and several derivatives. The derivatives are: transposon insertion mutant YM205 (■); strain YM205 harbouring the construct pRG315, which contains the intact pnp gene (□); and two merodiploid strains which contain an extra copy of *pnp* in their chromosome, i.e. YM607 (◇) and YM588 (○), derived from YM205 and W22703, respectively

However, the use of pRG315 to study the role of *pnp* in growth at low temperature had some drawbacks since, in contrast to the natural situation, harbouring of pRG315 leads to multiple copies of the *pnp* gene. In addition, copy number and plasmid stability may vary at different temperatures, introducing miscellaneous effects on *pnp* expression. Therefore, we introduced a second copy of *pnp*, including the 330 base pairs upstream region, into the chromosome of YM205, by means of homologous reconmbination with the construct pRG547, and studied growth characterisitics and *pnp*-expression in merodiploid strains with the desired integration. The suicide vector pKNG101 contains the *E. coli str*A and *str*B genes, conveying streptomycin resistance, and the *B. subtilis sac*B and *sac*R genes, which are lethal to their host when strains are grown in the presence of sucrose. Four exconjugants were selected for further analysis and all four strains were unable to grow in the presence of 5% sucrose, indicating that they contained a copy of pRG547. Analysis by PCR, using primers deduced from Tn5, *rpsO* and

pnp, and by Southern hybridization, using both transposon- or *pnp*-derived probes, showed that all four exconjugants had the desired constellation (Figure 6.8, upper part).
Growth characteristics at 5°C and 30°C were determined, and it appeared that the insertion of pRG547 had fully restored the psychrotrophic phenotype in all four mero-diploid strains: they showed wild-type growth rates at both 30°C and 5°C (the growth profile at 5°C of one of these strains, i.e. YM607, is shown in Figure 6.7). In addition, the PNPase production in YM607 was determined and it appeared to be restored to wild-type levels too, including the relative increase of PNPase at 5°C compared to 30°C (Figure 6.6: lanes 9 and 10, and Table 6.3). This indicated that a cold-inducible promoter is located in the 330 base pairs upstream region.

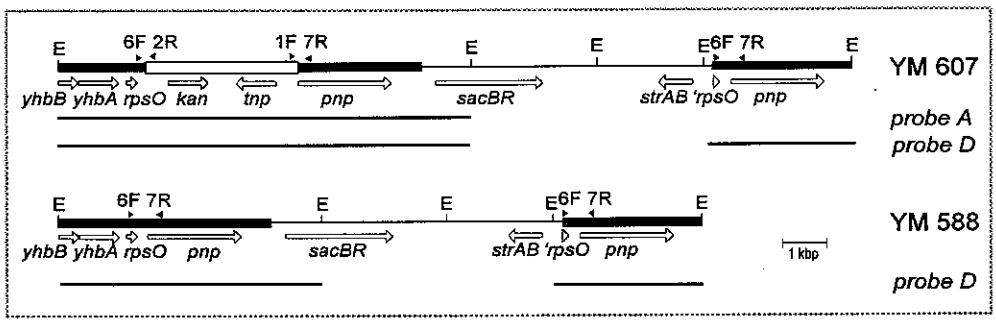

Figure 6.8 Genetic and physical map of the *rpsO-pnp* region of derivatives of *Y. enterocolitica* W22703 which harbour a duplication of *pnp*.

Upper part: A second copy of *pnp*, including the intercistronic region between *rpsO* and *pnp*, was introduced into the chromosome of the psychrotrophy defective (PD) mutant YM205 by homologous recombination with pRG547, resulting in strain YM607. *Yersinia* DNA is shown as a heavy line, the suicide vector DNA is shown as a thin line, and the transposon is depicted as an open bar. The position and orientation of relevant genes and primers are indicated by open arrows and by arrowheads (▸, ◂), respectively. The restriction sites for *Eco*RI are indicated by an E. Below the map, thin lines represent the fragments which hybridized in Southern analysis to probes A and D, respectively.

Lower part: A similar procedure as described above was used to insert a second copy of *pnp* into the wild-type strain W22703, resulting in strain YM588. All symbols are as indicated above. No fragments of YM588 hybridized to probe A.

6.3.5 Gene-dose Effect of *pnp* in the Wild Type Strain

Our results clearly implicated *pnp* in growth at 5°C. To investigate a possible gene dose effect on PNPase levels and/or the growth rate at 5°C, we constructed merodiploid strains which harboured two intact copies of *pnp* in their chromosome, by integration of pRG547 into the W22703. Four sucrose-sensitive exconjugants were identified, and tested for presence of the

desired insertion by PCR- and Southern analyses, as described above for YM205-derivatives. All four exconjugants appeared to have the desired constellation, i.e. to contain two intact copies of *pnp* (Figure 6.8, lower part).
The growth rates of the four exconjugants at 5°C and 30°C appeared to be indistinguishable from that of the parental strain, at both 5°C and 30°C (Figure 6.7). The level of PNPase was determined in one of the exconjugants (Table 6.1: YM588), and was found to be 1.6-fold higher at 5°C compared to 30°C, similar to the increase that was observed in the parental strain (Figure 6.6: lanes 11 and 12; and Table 6.3). No significant difference were found in the PNPase levels in strains that contained either one or two intact copies of *pnp* (i.e. W22703 versus YM588). Thus, no gene dose effect was observed, suggesting that *pnp* is autogenously regulated.

6.3.6 Analysis of the Promoter Region of the *pnp* Gene

Our results sofar indicated that *pnp* in *Y. enterocolitica* is under control of a cold-inducible promoter, which is most likely located in the region that ranges from the 3'-terminus of *rpsO* to the translational startpoint of *pnp*. Sequence analysis of this region revealed the presence of a 5'-ATTGG-3' motif at 230 bp upstream of the *pnp* translation startpoint (Figure 6.9).
In *E. coli*, this motif is present in several cold-inducible genes and acts as a binding site for the transcription-activator CspA [LaTeana *et al.* 1991; Jones *et al.* 1992]. A 5'-ATTGG-3' motif has also been found in the *pnp* promoter of the psychrotrophic bacterium *P. luminescens* [Clarke & Dowds 1994], but not in the major *pnp* promoter of *E. coli* [Régnier *et al.* 1987]. Comparison of the *rpsO-pnp* intercistronic region of *Y. enterocolitica* with the corresponding regions of *E. coli* and *P. luminescens* revealed a striking conservation of two stretches of approximately 20 base pairs (Figure 6.9: positions 1895-1919 and 1945-1964). In the mRNA of *E. coli* and *P. luminescens,* these stretches can form a double stranded stem that contains an RNase III cleavage site, as part of a stable secondary structure [Portier *et al.* 1987; Clarke & Dowds 1994], and a very similar structure, including the RNase III site, was deduced for *Y. enterocolitica* (Figure 6.10).

To investigate the role of the ATTGG-motif in cold-induction of the *pnp* gene, a derivative of pRG315 was constructed on which one of the thymidine residues was deleted from this motif in the *pnp* promoter region. The construct was multiplied in *E. coli* and then transformed to YM205. This procedure was expected to result in a strain that showed temperature-independent expression of *pnp*. However, despite several attempts using varying amounts of the pRG315-derivative, we did not succeed in the isolation of transformants of YM205 that harboured this construct, presumably because it was deleterious for the host (data not shown).

```
           1754                                                      1802
           |                               ***                          |
Y. ent.    GATCGAACGT  CTGGGTCTGC  GTCGCTAAGT  CT-GCGAGTT  TCGGGAGAAA
E. coli    C.....G..C  ..........  ........T.  ..T.......  ..A.A.A.GG
P. lum.    .ATTGGG...  ..T..CT.A.  ........-.  .AA..A....  ..A.TG....

           1803                                                      1849
           |                                                            |
Y. ent.    GGGCCAA---  ATTGGCCCCT  TTTTTCTAAG  AAGCAATGAT  AAAATACGCT
E. coli    .....TG---  .G........  ......A.GC  TGA.GGCAGC  ..TTC..TGG
P. lum.    ...G.C.TAT  ..G.C...T.  ..C....G..  ..ATC..C..  .TGTAG.AGC

           1850                                                      1899
           |                                                            |
Y. ent.    AGTATATTGT  TGTTCTATAT  TTTTAAAACA  TAGGATCTTC  CGGTGCAGAG
E. coli    .AAC..A...  --------..  .G..GCT.TG  A.T.......  ..T.......
P. lum.    .TA.G.A..C  .AA.GATG..  ....GGGT--  A...C.....  G.T.A.....

           1900                                                      1945
           |                                                            |
Y. ent.    GTTCGC[GCGG  CTAATG]AGAG  ACTTTAAC--  --TCATGGGG  GTTAAGGGTT
E. coli    ..........  ..........  G.....C.CA  CA.AGA.CT.  .G.T......
P. lum.    ..........  ..........  TGC....A--  --------..  AA.G..ACC.

           1946                                                      1995
           |                                                            |
Y. ent.    GT[CATTAGTC  GC]GAGGATGC  AGTGTGAAGG  TATGAGTAAC  AGGTGGATGA
E. coli    ..........  ..........  GCA.AAG.TC  GGGT.T....  .CCA.TG---
P. lum.    ..........  .........T  GCA-.....A  G..C.T.C.T  T.T..TC..-

           1996                         ↓                            2045
           |                                                            |
Y. ent.    GATCGTCAAT  ACGATCTGGC  ACCGCATAGT  ATAAGGATAC  TATTTTGCTG
E. coli    ------.CG.  .A.G.ACT.T  CTAAG.A..A  GA.......T  ..CA.....T
P. lum.    -------CTG  .AT..AAAAG  CAACGT..AC  GA...A...T  ..........
```

Figure 6.9
Nucleotide sequence of the intercistronic region between *rpsO* and *pnp* in *Y. enterocolitica* , and allignment with the corresponding regions of *E. coli* [Régnier *et al.* 1987] and *Photorhabdus luminescens* [Clarke & Dowds 1994].
The *Yersinia* DNA is numbered according to the sequence submitted to the EMBL/GenBank submission (accession nr. Y10692). Dots in the sequences of *E. coli* or *P. luminescens* indicate sequence identity with *Y. enterocolitica* DNA, and dashes indicate gaps introduced in the alignment to increase the number of matches. The stop codon of *rpsO* is indicated by stars and the translational start codon of *pnp* is underlined. The transposon insertion site in the PD-mutant YM205 is indicated by an arrow. The 5'-ATTGG-3' cold-shock motifs, as present in *Y. enterocolitica* and *P. luminescens*, are printed in bold. Boxes are around the conserved stretches that can form the double-stranded stem with the putative RNase III cleavage in the transcript, as depicted in Figure 6.10.

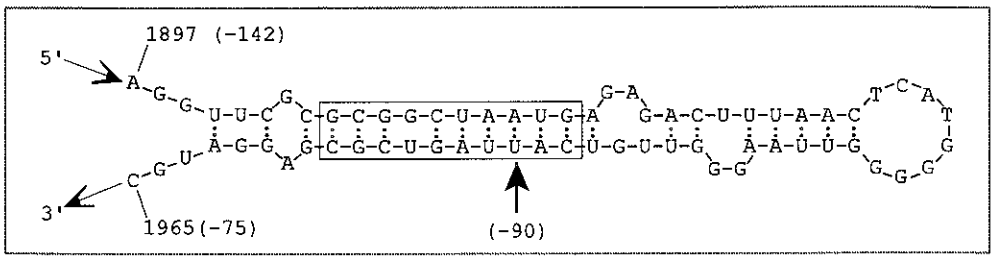

Figure 6.10
Potential secondary structure of transcripts from the *Y. enterocolitica rpsO-pnp* intercistronic region, predicted on the basis of sequence similarity with *E. coli* and *P. luminescens*. The numbers at the 5' and 3'-ends of the depicted stem-loop structure correspond with the location of these nucleotides in Y10692. Numbers between brackets indicate the positions in relation to the translation start codon of *pnp*. The putative RNase III processing site is marked by an arrow. The region surrounding the putative RNase III cleavage site which has an identical primary sequence in *Y. enterocolitica, E. coli* and *P. luminescens,* is boxed.

6.4 DISCUSSION

PNPase is required for growth at low temperature

This chapter documents the isolation and characterization of transposon-induced mutants of *Y. enterocolitica* that are severely restricted in growth at 5°C but not at 30°C (designated psychrotrophy-defective, PD), as well as the identification of *pnp* as the affected gene. In PD-mutants, the transposon was inserted upstream of the *pnp* gene, in a region which has a similar genetic organization as the *E. coli rpsO-pnp* locus. The transposon insertion mapped at the same site in all five PD-mutants isolated, i.e. sixteen base pairs upstream of the predicted *pnp* translational start point, suggesting that they were derived from a single insertion mutant. The insertion upstream of *pnp* was found to affect expression of *pnp*, as no specific mRNA or gene product, PNPase, could be detected in the PD-mutants. In a strain that was hampered in growth at both 5°C and 30°C (designated semi-PD), by contrast, the transposon insertion was not located in the *rpsO-pnp* operon and wild-type expression of PNPase was observed. Introduction of a second copy of *pnp* into the chromosome of a PD-mutant restored both PNPase production and the ability to grow at 5°C to wild-type levels. We concluded that PNPase is required for growth of *Y. enterocolitica* at 5°C, but not at 30°C. The PD-mutation was, for the greater part, also neutralized by trans complementation with *pnp* and this result supports the assumption that the PD-phenotype is mainly due to a polar effect of the transposon insertion on *pnp* expression. The somewhat slower growth of trans complemented PD-mutants, compared to the wild-type strain and the chromosomally complemented mutants, may be the result of metabolic stress due to harbouring of a multi-copy plasmid, a phenomenon

that has been observed previously in *Y. enterocolitica* [Goverde *et al.* 1994]. Nevertheless, the possibility cannot be excluded that the transposon insertion also affects the expression of genes located downstream of *pnp* which may also be involved in growth at low temperature. Possible effects of the transposon on genes located upstream of *pnp*, such as *rbfA*, are unlikely to be responsible for the PD-mutation because all revertants still contained the transposon insertion.

Pnp-expression is temperature dependent

PNPase belongs to a class of proteins, designated cold shock proteins, which are upregulated by *E. coli* upon a shift to lower temperature [Jones *et al.* 1987]. In *E. coli*, the *pnp* gene is adjacent to and partly cotranscribed with *rpsO*, a gene that encodes the ribosomal protein S15 [Takata *et al.* 1985; Régnier & Portier 1986]. The role of PNPase in the metabolism of *E. coli* has been studied thoroughly and is now believed to lie mainly in exonucleolytic mRNA degradation [Grunberg-Manago 1963; Littauer & Soreq 1982]. *In vitro*, PNPase non-specifically catalyses the reversible phosphorolytic reaction 'RNA + nPi ↔ n {nucleotide-5'-diphosphate}'. *In vivo*, the final breakdown of mRNA to the mononucleotide level is dependent on two exoribonucleases, PNPase and the hydrolytic enzyme RNase II. These enzymes can substitute for each other to degrade RNA, and the presence of either one of these enzymes is required for cell viability and mRNA turnover [Donovan & Kushner 1986]. In extracts from *E. coli* grown at 37°C, the mode of RNA degradation was shown to be predominantly hydrolytic whilst PNPase was responsible for only 10% of the total processive 3'→5'RNA degradation [Deutscher & Reuven 1991]. An important difference between PNPase and RNase II is that degradation of mRNA by the former is less energy consuming. This may explain the cold shock induction of PNPase in *E. coli* since energy levels have been shown to be growth limiting at low temperature and bacterial cells expend a significant amount of energy in mRNA turnover [Pao & Dyess 1981]. This assumption is supported by the finding that acclimatized growth at low temperature also affects PNPase: in the psychrotroph *P. luminescens*, the *pnp* gene is expressed 2 to 4-fold more at 9°C then at 28°C [Clarke & Dowds 1994] and in the present report it was shown that *pnp* expression is temperature-dependent in *Y. enterocolitica* too (± 1.6-fold more at 5°C then at 30°C). Interestingly, PNPase-deficient mutants of *E. coli* and *Bacillus subtilis* appeared to be restricted in growth at room temperature [Luttinger *et al.* 1996] and, in addition, more sensitive to antibiotics, such as tetracyclines [McMurry & Levy 1987; Wang & Bechhofer 1996]. This increased susceptibility is thought to be a consequence of as yet undefined alterations in the cell wall- or the cytoplasmic membrane, resulting from defective expression of genes involved in cell wall synthesis after the loss of PNPase activity. We have previously reported that *E. coli* and *Y. enterocolitica* use different strategies to cope with decreasing growth temperatures, with regard to maintaining membrane fluidity [Goverde *et al.* 1994] and it is not unlikely that the PD-mutants in the present study are altered with respect to this type of adaptation. Future investigations concerning the physiology of the PD-mutants might elucidate whether and how PNPase activity is linked with membrane composition and susceptibility to antibiotics.

Regulation of *pnp*-expression

What role does *pnp* expression play in the differences between *E. coli* and psychrotrophs like *Y. enterocolitica* or *P. luminescens*, with respect to their lower limits of growth temperature, and how is this role accomplished? In *E. coli*, *pnp* expression is under translational autocontrol and the first step in this autocontrol is the sequence-specific cleavage of the *pnp* message by the endoribonuclease RNase III [Robert-LeMeur & Portier 1992]. The specific RNase III cleavage site is located in a stable stem-loop structure at the 5'-end of the *pnp* message. A very similar secondary structure, with an identical RNase III site, has been observed in the mRNA of the *P. luminescens pnp* gene [Clarke & Dowds 1994] and our results indicate that an RNase III site is also present in the *Y. enterocolitica pnp* message. The striking conservation of the RNase III cleavage site in the *pnp* mRNA of both mesophilic and psychrotrophic bacteria suggests that this site is not involved in growth at low temperatures. The occurrence of an RNase III processing site in the *pnp* m-RNA of *Y. enterocolitica*, as well as the absence of a *pnp* gene dose effect, indicate that *pnp* expression is autoregulated at the translational level, as is observed in *E. coli*. Therefore, it seems unlikely that the temperature-dependent regulation of *pnp* in *Y. enterocolitica* is accomplished by RNase III.

Possible role of the ATTGG-motif

Several lines of evidence suggest that cold-adaptation of *Y. enterocolitica* requires cold-induced expression of *pnp*, which is regulated via a cold-inducible promoter and an ATTGG-recognition-site for a, as yet unknown, transcription-activating factor. First, the expression of several cold-inducible genes in *E. coli*, including *cspA*, *gyrA*, *hns* and *nusA*, is substantially increased upon overproduction of the major cold shock protein CspA, suggesting that CspA acts as a transcriptional enhancer of certain genes in the cold shock regulon [Jones *et al.* 1992]. The promoter regions of these cold-inducible genes of *E. coli* contain specific binding sites for CspA and the consensus sequence of these binding sites has been identified as 5'-CCAAT-3', or its inverted repeat 5'-ATTGG-3' [LaTeana *et al.* 1991; Qoronfleh *et al.* 1992]. In the two major *pnp* promoters, by contrast, these cold shock motifs are not found and *pnp* expression is not affected by over-expression of CspA [Jones *et al.* 1992]. However, the *rpsO-pnp* operon is located downstream of the cold-inducible *metY-nusA-infB* operon and polycistronic transcripts have been reported that cover the entire cluster from *metY* to *pnp* [Grunberg-Manago 1987]. It is therefore thought that the relative over-expression of PNPase in *E. coli* after a cold shock is due to an increased level of read through from the *metY-nusA-infB* operon into the *rpsO-pnp* operon [Sands et al. 1988]. Second, it was speculated that one feature that enables *P. luminescens* to grow in a cold environment is the evolutionary selection of a promoter directly upstream of *pnp* that is positively regulated at low temperatures, because PNPase is a cold acclimation protein in this organism and the *pnp* promoter contains the cold shock motif ATTGG [Clarke & Dowds 1994]. Third, a further indication that cold-active promoters might be involved in adaptation to low temperature has been found in a psychrophilic *Vibrio* sp.: this organism

expresses two structurally and genetically different isozymes of isocitrate dehydrogenase and one of these, IDH-II, is extremely thermo-labile and is encoded by a gene that contains a CCAAT-motif in its promoter region [Ishii *et al.* 1993].

In conclusion

In the present study, we found that *pnp* is cold-inducible in *Y. enterocolitica* and we showed that a 0.33 kbp region upstream of *pnp* is sufficient to retain its cold-induction. We identified an ATTGG motif at 0.23 kbp upstream of the PNPase translation start point and showed that insertion of a transposon just upstream of the first translated codon disrupted both *pnp* expression and the ability to grow at 5°C. These results strongly suggest that, in *Y. enterocolitica*, expression of *pnp* is under control of a cold-inducible promoter located in the *rpsO-pnp* intercistronic region. In an additional experiment (results not shown) we observed that neither the expression of *pnp* nor the growth at low temperature was affected by an insertion of 4.5 kbp DNA at a site 0.33 kbp upstream of *pnp*, which further indicates that in *Y. enterocolitica*, unlike the situation in *E. coli*, cold-induced *pnp* expression is not dependent on transcriptional read-through initiated upstream of *rpsO*. Apparently, *Y. enterocolitica* has, like other psychrophilic and psychrotrophic bacteria, facilitated its adaptation to low-temperature growth by selecting for the promoter of an important gene to be sensitive to a positive transcriptional regulator. However, our attempts to show that this sensitivity resides in the ATTGG-motif were thus far unsuccessful. Future experiments should answer questions regarding the role of the ATTGG-motif and the mechanism of transcriptionally regulated *pnp* expression in psychrotrophy of *Y. enterocolitica*.

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7

PNP-expression & Cold-Adaptation

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Analysis of *pnp* transcription regulation in psychrotrophic bacteria of the genus *Yersinia*: evidence for constitutive and cold-inducible promoters

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ABSTRACT

In the psychrotrophic bacterium *Yersinia enterocolitica*, polynucleotide phosphorylase (PNPase) is required for growth at low temperature, and expression of the *pnp*-gene was suggested to be under control of a cold-inducible promoter [Goverde *et al.* 1998]. In the present study, transcription initiation analysis of *pnp*-mRNA obtained from cells grown at either 30°C or 5°C, revealed the presence of two putative *pnp* promoters in the *rpsO-pnp* intercistronic region. According to the obtained primer extension signals, promoter P_{91}, initiating transcription at position -91 upstream of the structural gene, is not temperature-dependent, whereas promoter P_{197}, initiating transcription at position -197, is cold-induced. As a result, the level of *pnp*-mRNA was 5-fold enhanced at 5°C compared to 30°C. The -35 region of P_{197} appeared to overlap the previously identified putative cold-shock motif ATTGG. The cold-inducibility of gene expression in *Y. enterocolitica* via its *pnp* promoters was corroborated by enhanced levels of chloramphenicol acetyl transferase, at 5°C compared to 35° C, in cells which harbour a *cat*-gene under control of the *Y. enterocolitica rpsO-pnp* intercistronic region. DNA-sequence analysis of the corresponding regions from other Yersinia species revealed a 88.6-92.8% sequence identity with *Y. enterocolitica*. In addition, all Yersinia species appear to harbour one cold shock motif, either ATTGG or CCAAT, in this region.

Our data suggest that (i) *pnp*-transcription in *Y. enterocolitica* is under control of both a nearby constitutive promoter and a more remote cold-inducible promoter, and (ii) other, likewise psychrotrophic Yersinia species use a similar mechanism as *Y. enterocolitica* to regulate their *pnp*-expression at various temperatures.

7.1 INTRODUCTION

Yersinia enterocolitica is a psychrotrophic bacterium that is regularly implicated in human disease, either due to consumption of contaminated foods or drinks, or as a result from transfusion with contaminated blood products [Bottone 1997]. Preceding cold storage of these vehicles is frequently reported in outbreaks as well as in sporadic cases of yersiniosis. Hence, the ability of *Y. enterocolitica* to multiply at refrigeration temperatures highly contributes to the risk of this bacterium for human health. Recently, the expression of the *pnp* gene, encoding polynucleotide phosphorylase (PNPase), was shown to be indispensable for adaptation to low temperature of *Y. enterocolitica*: i.e. mutants in which *pnp*-expression was blocked by means of a transposon insertion just upstream of the structural gene were unable to grow at 5°C [Goverde *et al.* 1998]. Growth at 30°, by contrast, was not affected in this mutant. The ability to grow at 5°C was restored by introduction into the mutant of a parental DNA fragment that contained the *pnp* gene as well as 330 basepairs which precede the structural gene. Expression of *pnp* was shown to be cold-inducible in the parental wild type strain, as well as in the complemented mutant. The 330 bp region upstream of *pnp*, that comprised the 3'-terminus of the *rpsO* gene as well as the *rpsO-pnp* intercistronic region (Figure 7.1), was assumed to contain regulatory elements because (i) the expression of *pnp* was not affected in a mutant that contained a plasmid derived DNA-insertion at 330 bp upstream of the *pnp* translation start point, and (ii) the upstream region contains an ATTGG 'cold shock'-motif. In *Escherichia coli* and *Bacillus subtilis*, this motif, like its inverted repeat CCAAT, is a recognition site for their major cold shock proteins, CspA and CspB, respectively, which may act as transcriptional enhancers of several cold-inducible genes [LaTeana *et al.* 1991; Qoronfleh *et al.* 1992; Graumann & Marahiel 1994].

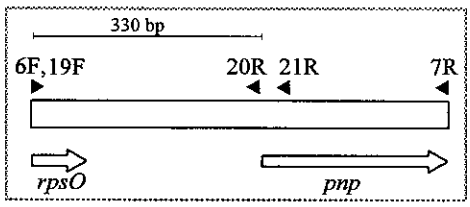

Figure 7.1
Genetic map of the *Y. enterocolitica* chromosome harbouring the 5'-terminus of *pnp*. The the position and orientation of the coding regions of *pnp* and the preceding *rpsO* gene are depicted by arrows, and relevant primers are indicated by arrowheads.

Several lines of evidence suggest that PNPase plays a role in cold-adaptation of various organisms. In *E. coli*, PNPase belongs to the class of so-called cold-shock proteins, which are upregulated upon a shift to lower temperature [Jones *et al.* 1987]. Surprisingly, none of the two major *pnp* promoters in *E. coli*, either located in the *rpsO-pnp* intercistronic region or upstream of *rpsO*, contains a cold-shock motif [Takata *et al.* 1985; Régnier & Portier 1986; Régnier *et al.* 1987]. However, *pnp* expression in *E. coli* might be under indirect control of a cold-inducible

promoter, i.e. via read through from the preceding *metY-nusA-infB* operon into the *rpsO-pnp* operon. Indeed, the *metY-nusA-infB* operon contains ATTGG and CCAAT motifs and polycistronic transcripts have been reported that cover the entire cluster from *metY* to *pnp* [Grunberg-Manago 1987]. Furthermore, a PNPase-deficient mutant of *E. coli* showed restricted growth at 16 and 23 °C and this 'cold-sensitivity' was also observed with a PNPase-deficient mutant of the mesophilic Gram-positive bacterium *Bacillus subtilis* [Luttinger *et al.* 1996]. Finally, PNPase was found to be cold-inducible, and to contain a ATTGG-motif in its promoter region, in the psychrotrophic insect pathogen *Photorhabdus luminescens* [Clarke & Dowds 1994].
In this study, we further characterized the *pnp* promoter region by mapping of the transcription initiation sites in *Y. enterocolitica*, by sequence analysis of the corresponding regions in other species of the genus Yersinia and by measuring of heterologous gene expression under control of the putative cold-inducible promoter.

7.2 MATERIALS & METHODS

7.2.1 Bacterial strains, Plasmids and Growth Conditions

Bacterial strains and plasmids used in this work are listed in Table 7.1. Cell lysates of *Y. pestis* strains were kindly donated by Dr. F. Reubsaet (RIVM, Bilthoven, the Netherlands). All other strains were grown overnight in NZ-broth (Bio101) at 30 °C, and frozen in 15% glycerol at -70 °C for permanent collection. To determine growth characteristics and to prepare samples for immunoblots, PCR, or primer-extension, strains were cultured in NZ-broth, in a shaking water bath, at 30 °C or 5 °C. Growth was recorded by measuring the optical density at 600nm (OD_{600}) at regular intervals. When required for cloning and selection, the media were supplemented with ampicillin (100μg/ml) or streptomycin (100 μg/ml).

7.2.2 Measuring of PNPase-levels

Broth cultures grown to mid-log phase (OD_{600} 0.6-1.0) were centrifuged (12000 x g) for 1 min and the cell pellets washed twice in phosphate buffered saline (PBS). When necessary, pellets were stored until use at -70 °C. Cleared cell lysates were prepared by sonication and successive centrifugation, as described previously [Goverde *et al.* 1998]. Similarly, protein concentrations were determined using BCA-reagent (Pierce) and the levels of PNPase were assayed on immunoblots, as described previously [Goverde *et al.* 1998].

7.2.3 Recombinant DNA Methods

General methods

DNA isolation, cloning of DNA fragments, transformations, plasmid isolations and agarose

gel electrophoresis were performed as described in [Ausubel *et al.* 1987]. Restriction enzymes were used according to the instructions provided by the manufacturer (Boehringer Mannheim, Germany or Gibco-BRL/Life Technologies, Gaithersburg, MD, USA). Transformations were performed by electroporation at settings: 2.5 kV, 200 W, 25 μF, using 0.2 mm cuvettes in a Gene Pulser apparatus (BioRad, Hercules, CA, USA).

Table 7.1 Bacterial strains and plasmids

STRAINS & PLASMIDS	CHARACTERISTICS / ORIGIN	REFERENCE / SOURCE
E. coli DH5α	*rec*A1, *lacZ*, host for pRG705	Gibco BRL
Y. enterocolitica		
W22703	plasmid-cured (pYV$^-$)	*3*
IP 134	origin unknown	Institut Pasteur[1]
*Ye*718	W22703 harbouring pRG705	*this work*
Y. bercovieri IP7506	origin unknown	Institut Pasteur
Y. frederiksenii BD 90-1052	origin unknown	RIVM[2]
Y. intermedia BD 80-683	origin unknown	RIVM
Y. kristensenii BD 90-650	from human faeces	RIVM
Y. mollaretti WA 751	origin unknown	Prof. Wauters, Univ. Leuven, Belgium
Y. pseudotuberculosis		
BD 86-280	from rabbit	RIVM
BD 90-1473	from human spleen	RIVM
Y. pestis		
IP Madagascar 73-93	origin unknown	Institut Pasteur
IP Madagascar 24-94	origin unknown	Institut Pasteur
IP Nathrang 63-115	origin unknown	Institut Pasteur
IP Hamburg 119	origin unknown	Institut Pasteur
IP Peru 184	origin unknown	Institut Pasteur
IP Kenya 164	origin unknown	Institut Pasteur
IP EV76	origin unknown	Institut Pasteur
EV76 CCUG32133	origin unknown	University Götenborg, Denmark
pKNG101	suicide vector, *strAB*	*11*
pUC21BM$^+$	cloning vector; ampicillin^R	Boehringer Mannheim
pRG315	pUC21BM$^+$ with insert *3'rpsO-pnp* from *Y. enterocolitica* W22703	*7*
pRG705	pUC21BM$^+$ containing *strAB* and W22703[*rpsO'-pnp'*]- *cat* fusion	*this work*

[1]Institut Pasteur in Paris, France
[2]National Institute for Public Health & the Environment, Bilthoven, the Netherlands

PCR & sequencing

Primers used for PCR-, sequencing- and primer extension reactions are listed in Table 7.2. PCR reactions were performed using a standard protocol which has been described earlier

[Goverde *et al.* 1998]. Template solutions were either appropriate dilutions of isolated DNA, or cellular lysates prepared by heating of a bacterial suspension in PBS for 20 min at 95°C and pelleting by centrifugation. PCR-reaction products were separated by agarose gel-electrophoresis and DNA-fragments of interest were purified from gel using QiaEx (Qiagen). DNA sequencing was performed as described earlier [Goverde *et al.* 1998] and the sequences were aligned using the Multalin software program.

Table 7.2 DNA sequences of primers used for PCR and sequencing

PRIMER	SEQUENCE[1]	GENE	POSITION in Y10692
Forward primers			
6F	CGTAAGCTGCTGGACTACCT	*rpsO*	1701-1720
19F	CGTAgGaTcCTGGACTACCTGAAGCGTA	*rpsO*	1701-1728
Reverse primers			
7R	GACGTGAAGTCAGTGTTTCG	*pnp*	2319-2300
20R	TAGTATCtcTAgACTATGCGGTGCCAGA		2037-2010
21R	TGTTGGCCGTACTGGAATTTA	*pnp*	2080-2060

[1] The sequences are given from 5' to 3', and the genes which harbour the sequences are indicated. The positions correspond with the numbering of the respective nucleotides in the sequence previously submitted to EMBL (accession number Y10692). Lower cast letters in the sequences of 19F and 20R indicate bases which differ from the DNA sequence in *Y. enterocolitica*, in order to introduce *Bam*HI and *Xba*I cloning sites in the amplification products.

Primer extension

The 5'-end of *pnp*-containing transcripts was mapped by means of primer extension, essentially as described in Sambrook *et al.* 1989. Briefly, total cellular mRNA was isolated from the strain *Y. enterocolitica* W22703, grown in NZ-broth to mid-log phase at either 30 or 5°C, using standard procedures. The synthetic oligonucleotide 5'-TGTTGGCCGTACTGGAATTA-3' (primer 21R) was 5'-end-labelled with 50 mCi ^{32}P-ATP. The labelled primer was hybridized to 30 mg target RNA, and c-DNA was synthesized using Superscript™RT (Gibco BRL). Samples were heated at 80°C for 5 min and run on a DNA-sequencing gel, next to sequencing markers generated by using the same primer and a plasmid containing the 6F-21R *rpsO-pnp* fragment as a template. The products were visualized by autoradiography and the intensity of the signals was quantified by densitometry using a GS-700 Imaging Densitometer (Bio-Rad).

7.2.4 Construction of a *pnp-cat* Promoter Fusion

A 0.8 kb DNA fragment containing a promoterless *cat*-gene block (Pharmacia/Biotech), encoding chloramphenicol acetyl transferase, was inserted at the *Hin*dIII site into pUC21BM$^+$ (Figure 7.2). The resultant plasmid was successively digested with *Xba*I and *Bam*HI and

ligated with a 0.4 kb *Xba*I/*Bam*HI digested PCR amplification product, obtained from pRG315 which comprised the *rpsO-pnp* intercistronic region of *Y. enterocolitica* W22703. A construct of the desired composition was identified on the basis of its *Eco*RI restriction fragment profile (pRG694). To generate a plasmid that could be maintained in *Y. enterocolitica*, a *str*AB gene block, obtained by *Xba*I/*Not*I digestion of pKNG101 and successive T4-treatment of the excised 1.8 kb fragment, was introduced into the *Sma*I-site of pRG694. A construct that contained the *str*AB insertion in the opposite direction as the *pnp-cat* fusion, as identified by its *Hin*dIII restriction profile, (pRG705), was introduced into *Y. enterocolitica* W22703. The transformants were isolated on selective agar plates, containing streptomycin. One transformant strain (*Ye*718) was chosen for determination of the *cat*-expression at 35 °C and at 5 °C.

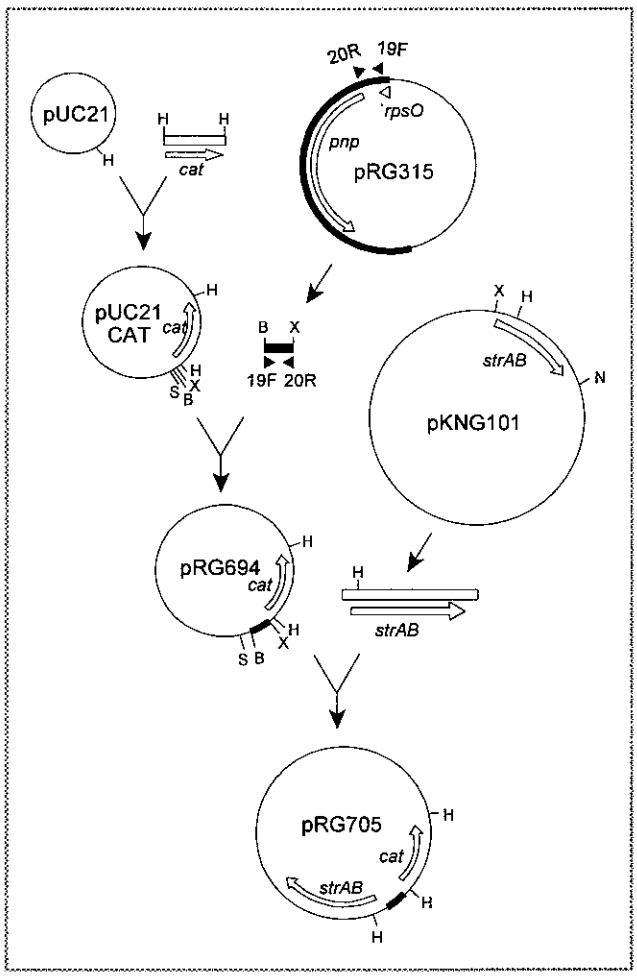

Figure 7.2
Construction of plasmid pRG705. A commercially available promoterless 0.8 kbp *cat*-genblock was inserted in the *Hin*dIII site of pUC21. The resulting construct, pUC21-CAT was digested with *Xba*I/*Bam*HI and ligated to a 0.4 kbp DNA fragment that contained the *rpsO-pnp* intercistronic region of *Y. enterocolitica*, which had been obtained from pRG315 by PCR amplification, using primers 19F and 20R, and subsequent digestion with *Xba*I/*Bam*HI. The resulting construct, pRG694, was digested with *Sma*I, treated with alkaline phosphatase and ligated with a blunt-end 1.8 kbp DNA fragment containing the *strAB* genes, which had been obtained from pKNG101 by excision with *Xba*I/*Not*I, and subsequent treatment with T4-DNA polymerase. DNA from *Y. enterocolitica* is shown by heavy black lines; the cloning vectors are depicted by thin lines. The position and orientation of relevant genes and primers are indicated by arrows and arrowheads, respectively. Restriction sites indicated are: B = *Bam*HI, H = *Hin*dIII, N = *Not*I, S = *Sma*I, and X = *Xba*I.

7.2.5 Measuring of *cat*-expression

Cell free extracts were prepared of broth cultures of strain *Ye*718, grown at 35°C or 5°C to an OD_{600} of 0.6-0.8, as described above for measuring of PNPase levels. Serial decimal dilutions containing 10 to 0.001 μg total protein/ml were assayed for the amount of chloramphenicol acetyl transferase, using a CAT ELISA kit (Boehringer Mannheim) and a Multiskan plate reader (Titertek), according to the manufacturer's instructions. To estimate the plasmid copy number ratio at 35 versus 5°C, chromosomal and plasmid DNA were isolated from approximately equal amounts of cells, and the respective DNA concentrations were estimated on agarose gel, by comparison with standard DNA solutions. The copy-number ratio was calculated from the plasmid concentrations, using the chromosomal DNA concentrations to compensate for different amounts of cells.

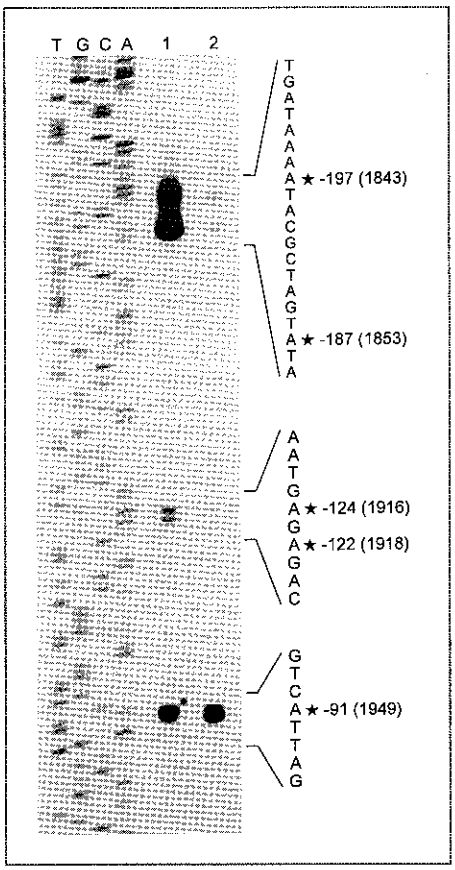

Figure 7.3 Primer extension mapping of the 5'-ends of *pnp* mRNA in *Y. enterocolitica*.
^{32}P-5'-end-labelled cDNA, obtained by primer-extension with a pnp-specific primer from total mRNA of W22703 grown at either 5 or 30°C, was run on a sequencing gel next to markers of the *rpsO-pnp* intercistronic region of W22703.
The lanes encoded with T, G, C and A contain the corresponding sequencing products of the *rpsO-pnp* intercistronic region; the primer exten-sion products of 5 and 35°C are in lanes 1 and 2, respectively. The bands were visualized by autoradiography.

7.3 RESULTS

7.3.1 Primer Extension Analysis of *Y. enterocolitica*

Depending on the growth temperature, various amounts of distinct *pnp*-transcripts were found. With the mRNA of cells grown at 30°C, the major primer extension signal corresponded with a transcript starting at -91 upstream of the structural gene (Figure 7.3: lane 2).

In addition, two very weak signals (together 10% of the total signal), were observed that corresponded with more remote transcription initiation sites, i.e. at positions -197 and -187. With cells grown at 5°C, however, the reverse was observed: i.e. the strongest signals corresponded with transcription initiation at -197 and -187, and a weaker signal was found at position -91 (Figure 7.3: lane 1: 42% , 39% and 15% of the total signal, respectively). In addition, two minor signals, corresponding to transcripts with 5'-termini at -122 and -124, were obtained at 5°C (2.5% and 2.2% of the total signal, respectively). The signals at position -91, which originated from equal amounts of target mRNA from cells grown at either 5°C or 30°C, appeared to be of equal intensity.

7.3.2 Other *Yersinia* Species

Growth characteristics

With the exception of *Y. pestis*, all strains were investigated for their ability to grow at 30 and 5°C. At 30°C, all strains grew, at approximately equal rates (data not shown). At 5°C, all strains grew, with growth rates that varied slightly among the species (data not shown).

Expression of *pnp*

All Yersinia strains (except *Y. pestis*) were grown at both 30°C and 5°C until mid-log phase and the respective levels of PNPase were compared on immunoblots. All strains showed enhanced levels of PNPase at 5°C compared to 30°C (Figure 7.4).

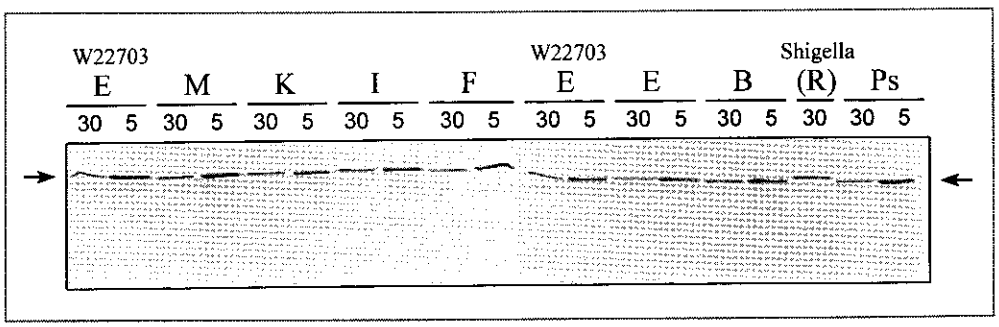

Figure 7.4 Effect of growth temperature on the PNPase levels in various Yersinia species. Equal amounts of total cellular protein, extracted from strains grown to midlog phase at 30°C or 5°C were analysed on a Western blot, using anti-PNPase polyclonal antiserum. Species and growth temperatures are indicated at the top of each lane: E = *Yersinia enterocolitica*, M = *Y. mollaretti*, K = *Y. kristensenii*, I = *Y. intermedia*, F = *Y. frederiksenii*, Ps = *Y. pseudotuberculosis* and B = *Y. bercovieri*. (R) refers to the initial erroneous biotyping of a *Shigella* strain as a *Y. ruckeri*. Numbers at the left side indicate sizes of standard proteins in a kilodalton. The position of PNPase is marked by an arrow.

Although the relative amounts varied among the species (i.e. at 30°C 70-130% and at 5°C 120-240% of the amounts observed with *Y. enterocolitica*), the cold-induced increase was more or less constant (i.e. 1.4 to 1.8-fold) (Table 7.3).

Table 7.3 Levels of PNPase in various Yersinia species at 30°C and 5°C

STRAIN	RELATIVE PNPase EXPRESSION (%)[1]		
	30°C	5°C	ratio 5°C/30°C
Y. enterocolitica W22703 (reference strain)	100 ± 10	160 ± 20	1.6
Y. enterocolitica	70 ± 10	120 ± 0	1.7
Y. bercovieri	100 ± 0	140 ± 40	1.4
Y. frederiksenii	130 ± 10	240 ± 50	1.8
Y. intermedia	130 ± 10	190 ± 40	1.5
Y. kristensenii	100 ± 20	150 ± 10	1.5
Y. mollaretti	110[2]	170 ± 0	1.5
Y. pseudotuberculosis	110 ± 10	150 ± 20	1.4

[1] Total cellular proteins, extracted from cells grown at 30°C or 5°C, were PNPase-specific immunostained on Western blots and the obtained signals were quantified by densitometry. The signal obtained with W22703 grown at 30°C was taken as a benchmark, and the corresponding PNPase level was arbitrarily set at 100%. The relative PNPase levels at 5°C, and in the remaining strains, were calculated from the corresponding densitometric signals.

[2] All experiments, except the one involving *Y. mollaretti* grown at 30°C, were performed twice.

7.3.3 Comparison of *rpsO-pnp* intercistronic regions

Sequence analysis of DNA-regions upstream of *pnp*

The *rpsO-pnp* intercistronic region of *Y. enterocolitica* was aligned to those of *E. coli* and *P. luminescens* and the alignment is depicted in Figure 7.5. The positions are indicated of the identified 5'-termini of *pnp* mRNAs in these three species. Putative promoters were found in the DNA regions upstream of the transcriptional start points -91 and -197, either by comparison with *pnp* promoters in *E. coli* [Evans & Dennis 1985] or with consensus eubacterial promoter sequences [Wösten 1998] (Figure 7.5: lines above the sequence).

Single amplification products, ranging in size from approximately 0.6 to 0.8 kb, were obtained with the lysates of all Yersinia strains, using primers 6F and 7R, (Figure 7.6: upper part), exceptfrom *Y. frederiksenii* and *Y. pestis*, which yielded one or more additional bands of various sizes (Figure 7.6: lower part). Only one of the *Y. pestis* lysates, i.e. strain IP-Hamburg-19, gave rise to a single amplification product of ±0.65 kb (Figure 7.6, lower part: lane 5). This fragment, as well as the 0.5-0.8 kb products obtained with all other Yersinia species, were isolated from the gel, and sequenced using primers 6F and 7R.

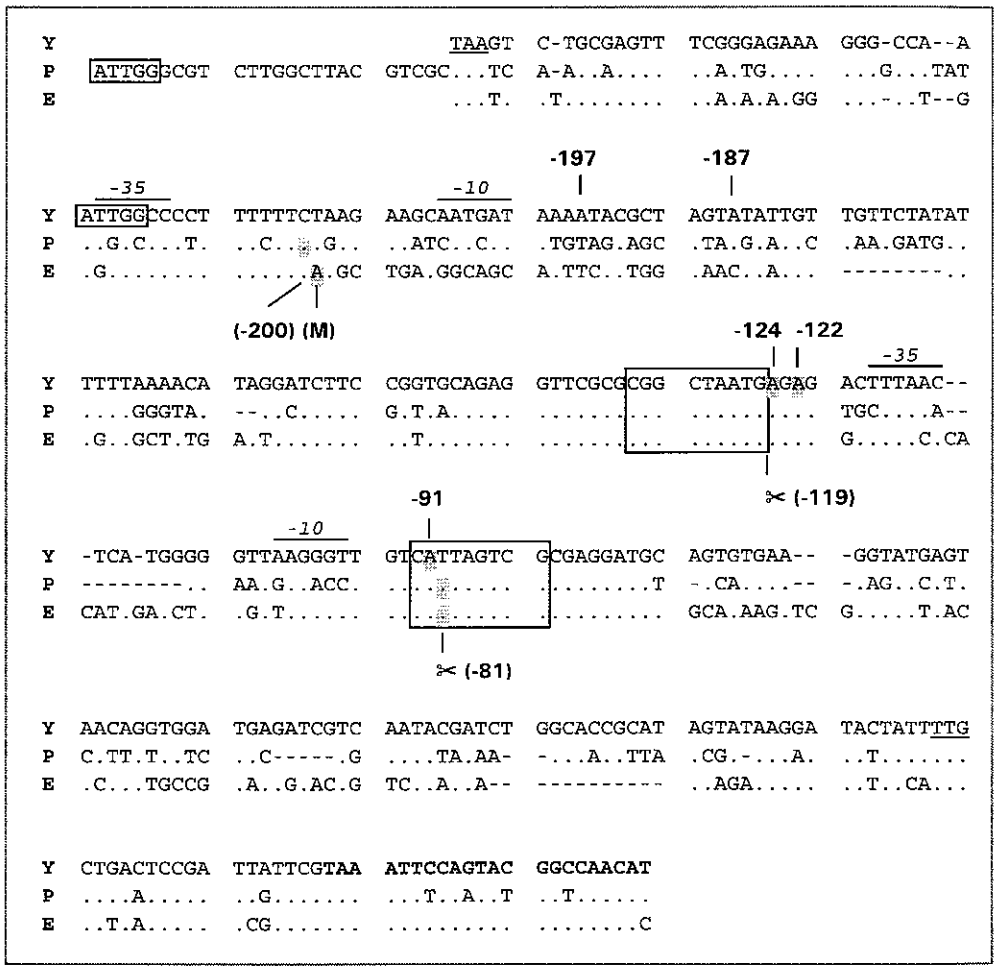

Figure 7.5 Identified and putative *pnp* transcription initiation sites and regulatory elements in the *rpsO-pnp* intercistronic DNA regions of *Y. enterocolitica*, *E. coli* and *P. luminescens*.
The data concerning *E. coli* and *P. luminescens* are from J02638 [Régnier & Portier 1986] and X76069 [Clarke & Dowds 1994], while *Y. enterocolitica* data are from this study. The translation stop codon of *rpsO* and the translation start codon of *pnp* are underlined. The region complementary to the primer used for primer extension (21R) is in bold typeface. The transcription initiation sites corresponding with the observed 5'-termini of *pnp*-mRNA's are shaded, and their positions in relation to the translational start points of the respective *pnp* genes are indicated (in bold) above (*Y. enterocolitica*) or below (*E. coli* and *P. luminescens*) the sequences. The -10 and -35 regions of the putative promoters in *Y. enterocolitica* are indicated by lines above the sequence. Boxes are around the cold shock motifs in *Y. enterocolitica* and *P. luminescens* and around the conserved stretches that can form a secondary mRNA structure. The mRNA maturation- and the RNaseIII processing-sites identified in *E. coli* are indicated by the letter M, and by scissors, respectively.

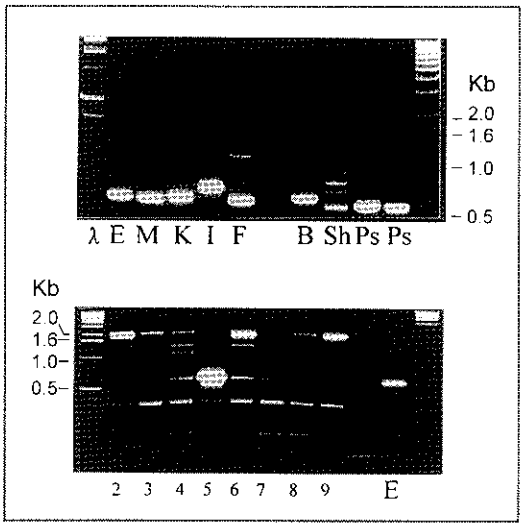

Figure 7.6
PCR-products of various *Yersinia* species, using primers derived from the *rpsO-pnp* intercistronic region of *Y. enterocolitica*.
Upper part: abbreviations used for *Yersinia* species are the same as in Figure 7.4.'Sh' refers to the *Shigella* species that was erroneously considered to be *Y. ruckeri*. The first and last lanes contain DNA size markers, and numbers at the right side indicate DNA sizes.
Lower part: products of *Y. pestis* strains are in lanes 2-9. *Y. enterocolitica* (E) was used as a control. The first lane contains a DNA size marker, and DNA sizes are indicated on the left side.

The sequenced DNA-fragments of the various species included the region that encoded the first 74 amino acids of PNPase, and this region revealed a 100% AA identity in all *Yersinia* species (data not shown).
The sequenced DNA-fragments also included the *rpsO-pnp* intercistronic regions. When these regions were aligned with the corresponding 264 bp region of *Y. enterocolitica* W22703 (GenBank accession no. Y10692: bp 1779 to 2042), a strong sequence similarity between all Yersinia species was observed (Figure 7.7). In *Y. enterocolitica* IP134, four additional bases were observed compared to Y10692 (98.5% identity), whereas the sequence identity with Y10692 in the other Yersinia species ranged between 88.6 and 92.8%. However, in *Yersinia intermedia*, *Y. bercovieri*, and *Y. kristensenii*, insertions of 121, 46 and 26 bps, respectively, were found at a site 180 bps upstream of the *pnp* translation start point.
Putative cold shock motifs were found in all Yersinia species, but three different patterns were observed: i.e. single ATTGG motifs were found at identical positions in *both Y. enterocolitica*, *Y. intermedia* and *Y. frederiksenii*, whereas at the corresponding sites in *Y. mollaretti*, *Y. kristensenii* and *Y. bercovieri*, single CCAAT motifs were found. In both *Y. pseudotuberculosis* and *Y. pestis*, single CCAAT-motifs were found at a site 80 bp more downstream compared to the other *Yersinia* species.

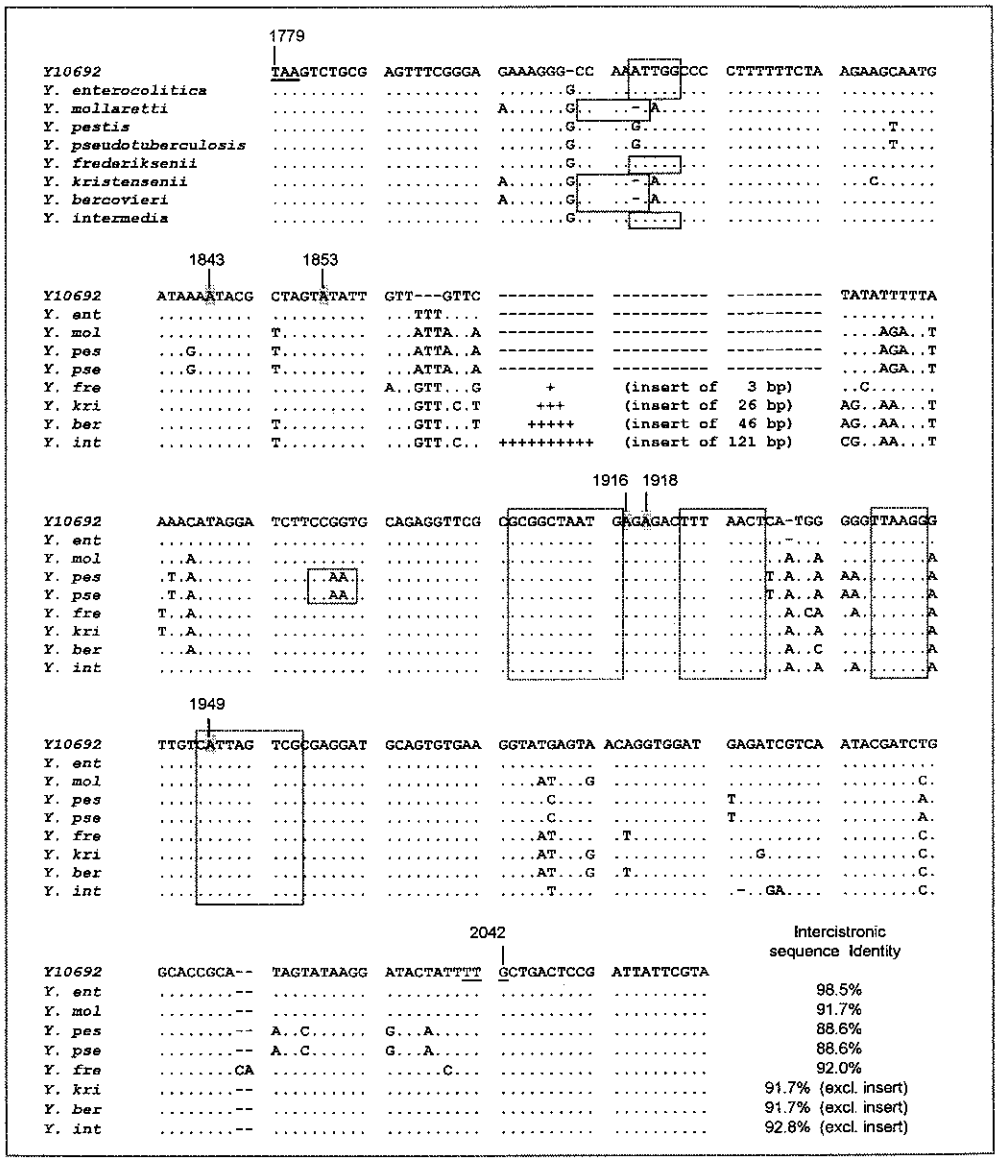

Figure 7.7 Alignment of the *rpsO-pnp* intercistronic DNA regions in the genus *Yersinia*.
The first line is part of the sequence of *Y. enterocolitica* W22703 (EMBL/Genbank nr. Y10692); the numbers above the sequence refer to the positions in Y10692. All other lines are sequences obtained in this study. The *rpsO* stop codon and the *pnp* start codon are underlined.The *pnp*-transcriptional start points are shaded. Dots indicate base-pair identity with Y10692; dashes indicate gaps introduced in the alignment to increase the number of matches. ATTGG- and CCAAT-motifs are boxed. Large boxes contain the stretches that can form a secondary mRNA structure.

7.3.4 Heterologous Gene-expression Under Control of the *pnp* Promoter-region of *Y. enterocolitica*

Heterologous gene-expression under control of the *pnp*-promoter-region was investigated using the *Y. enterocolitica* W22703 derivative *Ye*718, which harbours on plasmid pRG705 a transcriptional fusion between the *pnp* promoter and *cat*, as well as a streptomycin resistance gene. Cell free extracts from *Ye*718 grown at 5°C or 35°C in presence of streptomycin, and from the parental strain grown at the same temperatures, were assayed for the amount of chloramphenicol acetyl transferase (CAT). No CAT was found in the wild type strain W22703, whereas CAT expression was observed in *Ye*718 at both 5 and 35°C (Table 7.4). To our surprise, the CAT concentration appeared to be approximately 12.5-fold higher in the cells grown at 35°C compared to those grown at 5°C. The concentration of plasmid DNA, on the other hand, appeared to be 160-fold higher in cells grown at 35°C compared to 5°C. Thus, when standardized for equal amounts of plasmid DNA, the observed plasmid-encoded CAT-expression was approximately 12.8 fold higher at 5°C compared to 35°C.

Table 7.4 The effect of temperature on the *pnp*-promoter-controlled expression of *cat*.

T (°C)	MEASUREMENTS[1,2]		CALCULATIONS[3]		
	% CAT on total protein	plasmid DNA concentration (ng/10^{10}cells)	relative CAT contents	relative plasmid contents	relative CAT-expression per plasmid
35	12.5	250	100%	100%	1
5	1	1.56	(100x1/12.5=) 8%	(100x1.56/250=) 0.624%	(8/0.624=) 12.8

[1] Total protein isolates, extracted from the *Y. enterocolitica* YE718, harbouring a plasmid with a *pnp-cat* promoter-fusion, grown at either 35°C or 5°C, were assayed for chloramphenicol acetyl transferase (CAT) using a commercially available ELISA testkit.

[2] The amount of plasmid DNA isolated from equal amounts of cells was determined on agarose gels by comparison with standard amounts of DNA.

[3] To calculate the temperature-dependent *pnp* promoter-controlled CAT-expression, the CAT-expression at 35°C was taken as a benchmark and the corresponding contents of CAT and plasmid DNA were arbitrarily set at 100%. The relative contents of CAT and plasmid DNA in cells grown at 5°C, were calculated from the corresponding data. The relative CAT-expression per plasmid was calculated as the ratio of the relative CAT content versus the relative plasmid content.

7.4 DISCUSSION

Constitutive and cold-inducible *pnp* promoters in *Y. enterocolitica*

Our results demonstrated that transcription of the *pnp*-gene of *Y. enterocolitica* is controlled

by temperature: when starting from equal amounts of total mRNA prepared from cells grown at either 5 or 30°C, we obtained by *pnp*-specific primer extension approximately 5.4-fold more cDNA from cells grown at 5°C compared to 30°C (Figure 7.3). Unfortunately, the observed level of enhanced *pnp* transcription is not very consistent with our previous results, when we found 1.6 fold more *pnp* mRNA at 5°C compared to 30°C [Goverde *et al.* 1998]. At the moment, we do not have a satisfactory explanation for this dissimilarity, although it may partly be caused by differences in the growth phase of the cultures and in the techniques used.
The observation that at different temperatures transcripts of different length were present suggested that transcription is initiated by two different promoters, one of which is constitutive (P_{91}) while the other is induced at low temperature only (P_{197}).The presence of a constitutive promoter near position -91 is consistent with the presence of promoters at almost the same position in *E. coli* [Evans & Dennis 1985] and in *P. luminescens* [Clarke & Dowds 1994] (Figure 7.5). Similarly, a cold-inducible promoter near position -197 in Yersinia is consistent with the presence of a cold-inducible transcription initiation site at approximately the same position in *P. luminescens* [Clarke & Dowds 1994]. The transcript with its 5'-terminus at -187 was thought to be a degradation product of the transcript that initiates at -197. On the other hand, we could not exclude that at 30°C transcription is also initiated around positions -197 and -187, since some kind of post-transcriptional mRNA processing of those longer transcripts would also explain their virtual absence at 30°C. This would be consistent with the situation in *E. coli,* in which post-transcriptional RNaseIII processing of *pnp*-transcripts has been observed[Régnier & Portier 1986]. This processing depends on the formation of a strong secondary structure which covers the -130 to -80 region and contains two RNaseIII cleavage sites (Figure 8: -119 and -81, indicated by scissors). Such RNaseIII sites have also been identified in *P. luminescens* [Clarke & Dowds 1994]. Since the -130 to -80 *pnp* region in *Y. enterocolitica* shows a high degree of sequence similarity to the corresponding region in *E. coli* [Goverde *et al.* 1998], it is not unlikely that processing by RNaseIII also occurs in *Y. enterocolitica.* RNaseIII cleavage at the corresponding sites in *Y. enterocolitica* would indeed explain the faint primer extension signals which correspond with transcripts starting at -122 and -124, as were found in our experiments. On that line, it could be argued that transcripts with 5'-termini at -91, as were found at both 5 and 30°C, are similarly produced from transcripts starting at -197 by means of RNaseIII. In that case, reduced activity of RNaseIII at low temperature would explain why the original transcripts can still be found at 5°C but not at 30°C. In addition, it has been observed with *E. coli* [Takata *et al.* 1989] that unprocessed mRNA is much more resistant to degradation than processed mRNA. If this is also the case with *Y. enterocolitica,* the high levels of -197-187 transcripts in cells grown at 5°C might result from increased stability of unprocessed mRNA, especially if the activity of RNaseIII is reduced at low temperature. However, observations with *P. luminescens* argue against a significant effect of temperature-dependency of RNaseIII-activity [Clarke & Dowds 1994]. In this organism, much more of the

smaller transcript is found, compared to the longer transcript, regardless of the incubation temperature (28°C or 9°C). It is true that this phenomenon might also be explained as a primer-extension related artefact, caused by the tendency of reverse transcriptase to stop in regions of high secondary structure in the template RNA [Sambrook *et al.* 1989], but in that case we would expect to find a similar artefact in our experiments. However, we have foundthat the majority of *pnp* transcripts in *Y. enterocolitica* cells grown at 5°C start at positions -197 and -187, while the intensity of the signal at -91 is only 36-38% of these major signals. Finally, it is also possible that the transcripts with 5'-termini at -197/-187 result from endonucleolytic maturation of even larger transcripts, because such a maturation site has been observed in this region in *E. coli* (Figure 4, indicated by a letter M) [Régnier & Portier 1986]. However, we did not find *pnp* transcripts which initiate more than -197 bps upstream of the structural gene. This is consistent with our previous conclusion that in *Y. enterocolitica* there is no co-transcription of *pnp* and *rpsO* [Goverde *et al.* 1998]. Cold-induced transcription initiation at position -197 is further supported by (i) the presence of -35 and -10 motifs upstream of this site which resemble a consensus promoter sequence, and (ii) the overlap of the -35 motif with the ATTGG cold shock-motif.

Identical organization of the pnp-gene regulatory region in all Yersinia species

We found that all Yersinia strains that were tested were able to grow at 5°C and this is consistent with the literature [Gray 1995]. Due to absence of appropriate safety facilities, we did not grow *Y. pestis* strains, but this species has also been reported to be able to grow at 5°C [Dieudonné & Otto 1928; Sokhey & Habbu 1943]. With all Yersinia strains, PNPase levels were at least 1.4-fold enhanced at 5°C compared to 30°C, which is in accordance with our previous observations in *Y. enterocolitica* [Goverde *et al.* 1998]. This might implicate that expression of *pnp* is obligatory for growth at low temperature of all Yersinia strains investigated here. In addition, the overall high level of DNA sequence similarity and the presence of a cold-shock motif in all *pnp* regulatory regions, suggests that PNPase is involved in psychrotrophy of all Yersinia species, probably via an identical mechanism. It was observed in the *pnp-cat* promoter-fusion experiment that much less plasmid pRG705 could be isolated from cells grown at 5°C compared to 35°C, despite identical selective (streptomycin +) growth conditions and accounting for slightly differing culture densities. This effect indicates that the plasmid copy number is reduced in cells grown at low temperatures. It is tempting to speculate that a reduced copy is caused by a shortage in DNA-precursors at low temperatures, which enforces the bacterium to minimize the synthesis of extra-chromosomal DNA. This explanation is consistent with recent ideas about the major function of PNPase, i.e. to synthesize cytidinediphosphate (CDP) by RNA decay under energy-limiting conditions, thus coupling RNA-turnover to DNA-synthesis [Danchin 1997].

In conclusion, transcription of *pnp* in *Y. enterocolitica* is under control of a promoter that is strongly induced at low temperature. This induction is most likely regulated through overlap

with an ATTGG-motif, a feature that seems to be shared with all other species of the genus Yersinia. Further experiments, to elucidate the regulatory mechanism and to analyse the role of the PNPase at various temperatures, are in progress.

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8

Summary & General Discussion

ABSTRACT

It is known from the literature that:

- The application of chilling as a means of food preservation has frequently resulted in food borne infections with psychrotrophic micro-organisms, such as *Yersinia enterocolitica*, *Listeria monocytogenes* and *Aeromonas hydrophila*;
- The injurious effect on human health of an infection with *Y. enterocolitica* should not be underestimated because of the risk of serious post-infective complications;
- Almost all micro-organisms respond to an abrupt temperature down shift (within their growth range) by a temporary cessation of growth, and the concomitant transient synthesis of a set of specific proteins, called 'cold-shock-proteins';
- Many psychrotrophic micro-organisms synthesize at low temperatures a set of proteins, called 'cold-acclimation-proteins', whose expression is either enhanced or specifically induced at these temperatures;
- To explain the induction and function of the cold-shock response, a model called the Cold-Shock Ribosomal Adaptation (CSRA-) model has been proposed;
- This model comprises four steps, i.e. (1) cancelling of the regular translation of 'household' gene mRNA's, in favour of the translation of cold-shock gene mRNA's, (2) specific modification of the ribosome by the cold-shock proteins, (3) the re-initiation of regular mRNA translation, and (4) the concomitant downregulation of the expression of cold-shock genes;
- The CSRA-model does not explain why the production of cold-shock and/or cold-acclimation proteins does not cease in psychrotrophs;
- The inability of mesophiles to sustain growth at temperatures below 7°C has been explained as a result of insufficient cold-shock response, compared to psychrotrophs;
- Polynucleotide phosphorylase (PNPase), one of the two essential 3'-5'exoribonucleases in *E. coli*, is a cold-shock protein, but the gene is not cold-inducible by its own promoter. The other 3'-5'exoribonuclease, RNaseII, is not cold induced;
- PNPase is able to interact with the ribosome, and PNPase is better at the degrading of mRNA's which contain secondary structures, compared to its counterpart RNaseII;
- PNPase uses a relatively energy-saving mode of mRNA degradation, compared to RNaseII, and has in that way a central position in the efficient turnover of the essential DNA-precursor cytosine diphosphate (CDP) ;
- Energy levels are growth limiting for *E. coli* at low temperatures.

It has been shown in the present study that:

- The emergence of *Y. enterocolitica* as a human pathogen in our era can at least partly be explained as a result of the enormously increased application of refrigeration in the preservation of foods;
- The ability of *Y. enterocolitica* to grow at refrigeration temperatures is dependent on expression of the bacterial *pnp* gene, encoding the exoribonuclease polynucleotide phosphorylase (PNPase);
- The cellular concentration of PNPase in *Y. enterocolitica* is increased during growth at refrigeration temperatures;
- The cold-induced rise of the PNPase concentration in *Yersinia enterocolitica* is accompanied by an increase in *pnp* mRNA, and this is mediated by a cold-inducible promoter of the *pnp* gene;
- A similar cold-induced increase in PNPase concentration occurs in all other, invariably psychrotrophic, species of the genus Yersinia;
- In all other Yersinia species, the organization of the regulatory regions of the *pnp* gene is identical to that in *Y. enterocolitica.*

It is concluded that:

- PNPase is an essential factor for adaptation to reduced temperatures, presumably not only in *Y. enterocolitica*, but also in other, mesophilic bacteria;
- Due to its ability to interact with ribosomes, PNPase fits well in the CSRA-model. Its mode of action may be in the enhanced decay of those mRNA's which have lost their polysomes, resulting in a more efficient synthesis as well as in increasedtranslation of the mRNA's of the cold-shock genes;
- According to this function in the CSRA-model, PNPase might be a restrictive factor for efficient cold-adaptation. Consequently, an insufficient enhancement at 7°C or below, or a subsequent decline of PNPase expression, might be the cause of the definitive cessation of growth of mesophiles at this temperature;
- In view of the cellular difficulties in liberating sufficient energy under cold-stress conditions, it is not unlikely that the lower temperature limit for growth is set by the cellular capacity to maintain the catabolism under these conditions;
- On behalf of its energy-saving mode of mRNA decay, PNPase could be the key factor that is needed under energy-limiting conditions to provide the cell with the obligatory DNA-precursor cytosine diphosphate (CDP) ;
- Further studies are needed to unravel how *pnp*-expression is regulated and what role is played by PNPase in the process of cold-adaptation, in order to elucidate the mechanism(s) which set the lower temperature limit for growth of micro-organisms.

8.1 YERSINIOSIS AND THE 'COLD CHAIN'

The history of the gastro-intestinal disease known as yersiniosis dates back at least to the 1930s, but, as was described in **Chapter 1**, its break-through did not start before the early 1960s. Sepsis due to *Y. enterocolitica* after a blood-transfusion, on the other hand, was not reported before 1975. Since then, a rise has been seen in the occurrence of transfusion acquired *Y. enterocolitica* infections, especially in the highly industrialized countries.

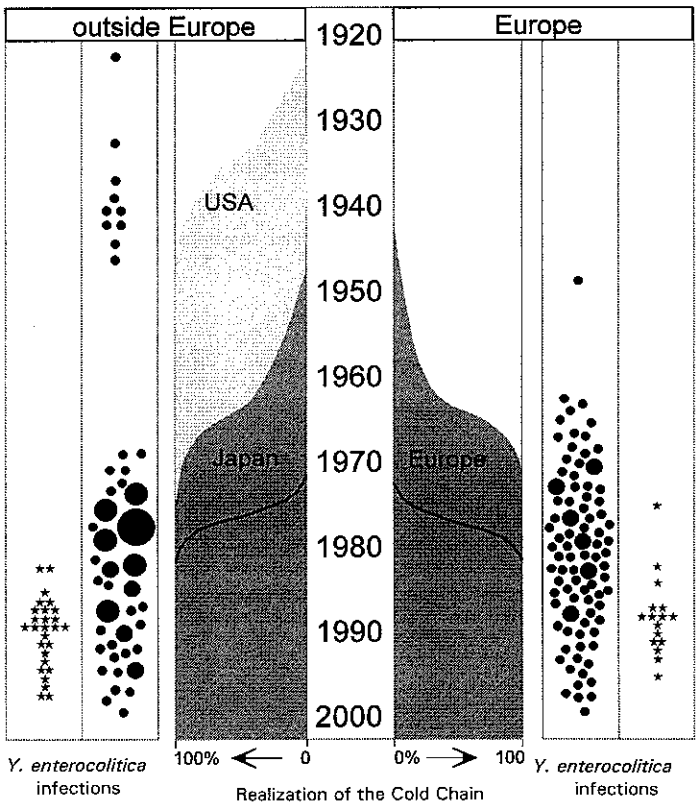

Figure 8.1
Schematic representation of the development of the Cold Chain, the increase of yersiniosis, and the generilization of prolonged cold storage of blood products, since the 1920s.
The realization of the Cold Chain is depicted by the grey shading in the center of the figure; the gradual switch to prolonged cold-storage of blood products is shown by a heavy line. The incidence of the disease yersionosis is depicted by dots: small dots refer to (numerous) sporadic cases, while the larger dots refer to outbreaks.
The blood tranfusion-acquired fatal infections with *Y. enterocolitica* are depicted by stars.

A survey of the literature concerning refrigeration, described in **Chapter 2**, shows that chilling has started its triumphal march in our era, after technological inventions had enabled the development of mechanical refrigeration, and when electricity had powered all the industrialized world. From the 1960s onwards, refrigeration has become a general means of keeping foods from spoilage at all stages between production and consumption. Likewise,

technological improvements in the 1970s enabled a prolonged storage of blood products at refrigeration temperatures, and storage for 28 days and more became common practice in the 1980s and 1990s. In the context of food- and blood storage at low temperatures, psychrotrophic pathogenic micro-organisms like *Y. enterocolitica* form a special problem. Their versatility with respect to growth temperature enables these organisms to react opportunistically at temperature fluctuations. Consequently, from a minor fraction in the natural microbial flora of foods, or hidden in macrophages in otherwise sterile blood-products, they might capture a dominant position during chilled storage and may eventually increase to infective amounts. As is schematized in Figure 8.1, the referenced literature shows that there are striking synchronisms in the rise of human yersiniosis on the one hand, and the shifts towards chilling as a general preservation method for foods and blood-products on the other hand. It may be concluded that the ability to grow at low temperature has indeed played an important role in the expansion of *Y. enterocolitica* as a human pathogen.

8.2 *Y. ENTEROCOLITICA*: A HUMAN HEALTH HAZARD

In the whole spectrum of microbial agents responsible for food borne human disease, the bacterium *Y. enterocolitica* only plays a minor role. Nevertheless, infection with this bacterium is not to be disregarded as a significant cause of morbidity and economic losses, due to a whole array of serious post-infectious complications (described in **Chapter 1**). Although the disease is most prominent in pig-breeding countries and among populations with a high pork consumption, its real incidence is largely unknown. Some trends may be perceived for countries with a reporting obligation, such as a decline in North-West Europe since the mid 1990s, which is thought to be due to improved pig-slaughtering techniques and household-hygiene. However, yersiniosis is not a notifiable disease in most parts of the world, and therefore the incidence data, as presented in Chapter 1, are generally not more then a rough indication for occurrence of the disease. In general, the true incidence of food borne disease is likely to be under-reported, since only a small proportion of persons with gastrointestinal complaints seeks medical attention [Adams & Moss 1995]. In order to compensate for under-reporting, multiplication factors ranging from 25 up to 350 have been suggested to estimate the actual amount of cases [Archer& Kvenberg 1985; Todd 1989a,1989b]. Although it is not known to what extent these figures are applicable to yersiniosis, it is not unlikely that the registered cases of yersiniosis represent less than 10% of the actual number of infections [Christensen 1987; Nesbakken 1992]. In addition to the under-reporting that may result from ignoring or overlooking clinical symptoms, the incidence of the disease might also be underestimated due to technical factors, such as poor isolation- or detection procedures. Cultivation at 37°C, for example, might easily lead to false negative results, either due to overgrowth by other organisms or to loss of the virulence plasmid. At the end of the 1980s, exclusive use of conventional isolation

procedures was common practice in many laboratories. At the time, most DNA-hybridization methods required specific equipment because of the use of radioactive labels, and the PCR-technique was still in its infancy. Therefore, this study was started by developing a rapid and easy method for detection of pathogenic strains of *Y. enterocolitica* in primary isolates of human and animal origin, applying a non-radioactive colony hybridization technique and targeting specific genomic sequences. Development and application of this method is described in **Chapter 4.** When applied to human clinical samples, there were no large discrepancies between the results with the newly developed method and conventional analysis. With samples of porcine origin, however, markers of virulent *Yersiniae* were detected in 12% of samples that were negative by cultivation.

It was concluded that yersiniosis is most likely of greater significance for common health than the figures of registered cases and hospitalization suggest. Poor isolation methods are not likely to contribute to underestimation of the incidence of human yersiniosis. Nevertheless, the use of modern, DNA-targeting methods is preferable to exclusive application of conventional methods when low levels of contamination are to be detected, such as might occur in raw and prepared food products.

8.3 COLD-ADAPTATION IN *Y. ENTEROCOLITICA*

As appears from **Chapter 3**, cold-adaptation of micro-organisms is reflected in the composition and structure of cellular proteins and lipids. As cold-adaptation must lie at the root of the ability to grow in the cold, we have tried to find key-factors for psychrotrophy by studying the differences in lipid- and protein compositions of various strains of *Y. enterocolitica* grown at temperatures ranging from 1 to 35°C. That part of our study is described in **Chapter 5.**

Membrane fatty acids

The most common cold-response for all micro-organisms, an increase of the unsaturation of membrane lipid acyl chains in order to retain a certain level of membrane fluidity, was also found in *Y. enterocolitica.* In addition, the second most common lipid adaptation, i.e. reduction of the average chain length, was also seen in *Y. enterocolitica.* These results were consistent with the results from other studies on the fatty acid compositions of Y. enterocolitica at various temperatures [Abbas & Card 1969; Nagamachi *et al.* 1991]. The situation in *Y. enterocolitica* contrasts with that in the closely related, but mesophilic, bacterium *Escherichia coli,* in which the average chain length slightly increases with temperature fall [Marr & Ingraham 1962]. The differences between *Y. enterocolitica* and *E. coli* with respect to their major fatty acid shifts are shown in Figure 8.2. *E. coli* adapts to cold by synthesizing increased amounts of C18:1, and this is accomplished, as described in Chapter 3, by the cold-induced enhanced activity of synthetase II compared to synthetase I in the C16-to-C18 elongation step.

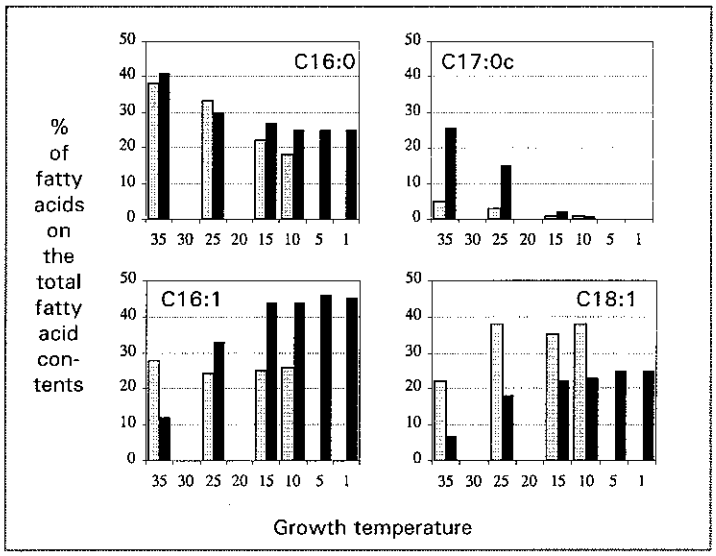

Figure 8.2
Changes in the relative contribution of the major fatty acid components in *Y. enterocolitica* and in *E. coli*, in response to growth temperature.
The black bars in the graphs represent the data of *Y. enterocolitica*; the grey bars are data from *E. coli*.
The type of fatty acid is indicated in the graphs.

It is possible that a similar two-enzyme system also regulates the change in unsaturation level in *Y. enterocolitica* between 35 and 15°C, but - if such a system is present - it is unlikely to be of great importance for cold-adaptation, since the levels of both C16:1 and C18:1 vary little between 15 and 1°C. It is not unlikely, on the other hand, that *Y. enterocolitica* has a temperature-regulated elongase enzyme similar to that in *E. coli*, which interconverts C16:0 and C18:0, in view of its high levels of C16:0, especially at 15°C and below. The striking difference with *E. coli* with respect to the amounts of cyclopropane fatty acids suggests that *Y. enterocolitica* converts large amounts of C16:1 into C17:0^c at enhanced temperatures and stops doing so when the temperature drops below 20°C. However, the physiological relevance of this behaviour is still unclear. In conclusion, marked dissimilarities exist in the fatty acid patterns of *Y. enterocolitica* and its close relative *E. coli*, but it is still to be unravelled whether these play a role in setting their minimal growth temperatures or whether these are just side effects of the difference in growth ranges.

Protein composition

As mentioned in Chapter 3, it has been known since the 1970s that the levels of most cellular proteins in *E. coli* do not change greatly within its normal temperature range (20-37°C). However, a few proteins exhibit increased levels near the lower temperature limit of growth, and at 13.5°C several temperature-specific proteins have been detected. This suggests a crucial role for these so called 'thermometer' proteins in bacterial adaptation to lower temperatures. This idea has gained further support by the discovery of specific 'Cold-Shock'-proteins (CSPs) in *E. coli*, which are produced when the cells are abruptly shifted from 37 to 10°C. However,

it had been shown in earlier studies that the synthesis of proteins stops in *E. coli* cells shifted to 5°C and that the cells cease growing due to a block in initiation of translation. Hence, the cold-shock response apparently reflected the adaptation to a significant drop in temperature, but its capacity seemed to be limited. The existence of 'thermometer-proteins', on the other hand, indicated that specific 'Cold-Acclimation'-proteins (CAPs) could be responsible for adaptation to a permanently cold environment. This assumption was supported by the observation that in the psychrotroph *Pseudomonas fluorescens,* the onset of protein synthesis was not affected by a temperature downshift to 5°C. In order to detect possible cold-acclimation proteins in *Y. enterocolitica*, we started to analyse the protein patterns obtained from cold-acclimatised cells and from cells grown at 'normal' temperatures. However, we only found minor differences in the protein patterns of cells grown at 1 or 5°C compared to 20 or 30°C, i.e. the presence of a ± 30 kDa protein at the lower temperatures only. The techniques used did not allow further characterization of this protein, so we did not gain clues about which enzymes were possibly involved in cold-adaptation. We therefore changed our strategy from studying an intact psychrotroph to the inhibition of psychrotrophy in such a strain, by transposon-induced mutations, and subsequent identification of the affected gene(s).

8.4 PNPASE: ESSENTIAL FOR GROWTH OF *Y. ENTEROCOLITICA* IN THE COLD

PNP mutants are cold sensitive

The random introduction of a transposon in the chromosome of a wild type, psychrotrophic strain of *Y. enterocolitica*, described in **Chapter 6,** resulted in several mutants that were specifically affected in their ability to grow at low temperature (designated PD, for psychrotrophy-defective). It turned out that all PD-mutants were deficient in expression of the *pnp* gene which encodes the 3'-5'-exoribonuclease polynucleotide phosphorylase (PNPase). However, none of these mutants was affected in growth at 30°C. This strongly suggested a stringent requirement of PNPase for adaptation to low temperature.

These results were consistent with observations in PNPase-deficient mutants of both *E. coli* and *Bacillus subtilis*, which had also been shown to be restricted in growth at reduced temperatures (23°C and 16.8°C, respectively). On the other hand, other *E. coli* mutants with a low level of PNPase activity have been described which grow poorly at 44°C, suggesting that PNPase might also be involved in adaptation to elevated temperatures [Krishna & Apirion 1973; Donovan & Kushner 1986]. Nonetheless, PNPase was unquestionably involved in growth of *Y. enterocolitica* at low temperatures, since the mutation could be complemented by the introduction into the mutant of a second copy of the *pnp* gene, including its putative regulatory region.

PNPase required for mRNA decay under energy limiting conditions?

The enzyme polynucleotide phosphorylase is widely distributed among bacteria, and is also found in many eukaryotic systems [Godefroy-Colburn & Grunberg-Manago 1972]. This apparent ubiquity suggests an important role in cell physiology, and this role is generally believed to lie mainly in exonucleolytic mRNA degradation. In *E. coli*, the final breakdown of mRNA to the mononucleotide level is accomplished by two 3'-5' exoribonucleases, i.e. RNaseII and PNPase, and the presence of either one of these enzymes is required for cell viability.

Today, several lines of evidence suggest that the functioning of PNPase comes into prominence under energy-limiting conditions, because mRNA breakdown by PNPase, which yields nucleoside diphosphates, is energy-saving compared to degradation by RNaseII, which produces nucleoside monophosphates. In view of this, it is important to realize that bacterial cells expend a significant amount of energy in mRNA turnover, and energy levels are growth limiting for *E. coli* at low temperatures [Pao and Dyess, 1981]. It is also known that, at 37°C, RNaseII is responsible for 90% of mRNA breakdown, whereas PNPase accounts for only 10% of the final decay [Deutscher & Reuven 1991]. In the soil bacterium *Bacillus subtilis*, on the other hand, RNaseII is absent and mRNA degradation is mainly accomplished by PNPase. It has been suggested that this difference reflects energy considerations, since a soil bacterium generally finds itself in an 'energy-poor' environment, especially in comparison with a micro-organism whose normal habitat is the large intestine of vertebrates. Unfortunately, it is not known whether or not *E. coli* switches to a higher contribution of PNPase in mRNA breakdown under energy-limiting conditions. However, the cellular PNPase concentration is transiently increased after a temperature downshift (it is one of the minor cold-shock proteins in *E. coli*), and an inverse relationship exist between the activities of PNPase and RNaseII [Zilhão *et al.* 1996]. With respect to the situation in *Y. enterocolitica*, information about the presence and/or activity of RNaseII is still lacking, but it appeared from our studies that PNPase is a cold-acclimation protein, a Cap, in this organism. In addition, we observed raised levels of both the *pnp* mRNA and the protein during growth at reduced temperatures, and this increase has also been observed in the psychrotrophic bacterium *Photorhabdus luminescens* [Clarke & Dowds 1994]. Thus, it might very well be that, independently of an organism's growth range, the increased synthesis of PNPase reflects a response to worsened energy conditions.

8.5 TRANSCRIPTIONALLY REGULATED, COLD-INDUCED EXPRESSION OF *PNP*

As has been described in **Chapter 7**, we have found a putative cold-shock induction motif (ATTGG) in the *rpsO-pnp* intercistronic region of *Y. enterocolitica,* and a promoter sequence which overlaps this motif was identified. Furthermore, we observed that at 5°C, but not at

30°C, *pnp* transcripts can be found which originate from this promoter. In addition, we have found that in all other, invariably psychrotrophic, Yersinia species cold-shock motifs (ATTGG or CCAAT) are present in the *pnp* promoter regions. Other investigators have previously showed that the corresponding region of the psychrotroph *P. luminescens* also harbours an ATTGG motif. In the mesophil *E. coli*, by contrast, the *pnp* promoters are devoid of cold-shock motifs, and its transient cold-induction is thought to be a result of transcriptional read-through from cold-inducible preceding genes [Sands *et al.* 1988]. This indicates that directly cold-inducible expression of *pnp* is a prerequisite for successful acclimation to low temperatures.

PNPase: an additional factor in the Cold-Shock Ribosomal Adaptation model?

The adaptation of micro-organisms to reduced temperatures is now generally thought to be dependent of CSPs and CAPs [Yamanaka *et al.* 1998], and the Cold Shock Ribosomal Adaptation model, as described in Chapter 3, has been proposed to explain the putative mechanism. In this model, the induction of most *E. coli* CSPs is regulated by a transcriptional enhancer, CspA, and this process is thought to be mediated through a cold-shock recognition motif (ATTGG or CCAAT) in or nearby the promoter of the genes in question.

The designers of the Cold-Shock Ribosomal Adaptation model thought that their model could only partially explain the cold-shock response in cold-adapted micro-organisms, because in these organisms, the production of CSP-like CAPs does not cease after the adaptation has been accomplished. Surprisingly, they did not specify a particular role for PNPase in their model although at the time 'cold'-sensitive *pnp* mutants were known, which indicated that PNPase is required for bacterial adaptation to reduced temperatures. Since then, however, other investigators have argued that PNPase might also function in facilitating translation re-initiation after a temperature down-shock, like most CSPs [Bycroft *et al.* 1997]. It was suggested that this might be accomplished by interaction with the ribosome, since the PNPase C-terminus is highly homologous to the ribosomal binding domain of the ribosomal protein S1 [Régnier *et al.* 1987]. Recently, it was shown that PNPase is indeed essential role for cellular cold-adaptation by affecting the ribosome metabolism [Zhou & Deutscher 1997]. Moreover, the earlier observation that re-initiation of translation does not occur when *E. coli* is chilled to 7°C or below, suggests that the attainable degree of cold-adaptation is probably set by the availability of sufficient amounts of ribosome-modifying proteins. Apparently, the amount of one or more of the ribosome-modifying proteins becomes restrictive at the lower temperature limit of growth. Our results strongly suggest that PNPase might be such an enzyme, because (i) PNPase is a crucial enzyme for cold growth of *Y. enterocolitica*, (ii) *pnp* transcription in *Y. enterocolitica* is enhanced at 5°C via a cold-inducible promoter, (iii) identical *pnp*-promoters are present in all other Yersinia species, and (iii) the levels of PNPase are enhanced in cold-acclimatised cells of all species of the psychrotrophic genus Yersinia. In other words, PNPase fits perfectly in the Ribosomal Cold-Shock model (Figure 8.3), while the difference in cold-inducibility of the *pnp* gene could explain why *Y. enterocolitica* can adapt to 7°C and below, whereas *E. coli* cannot.

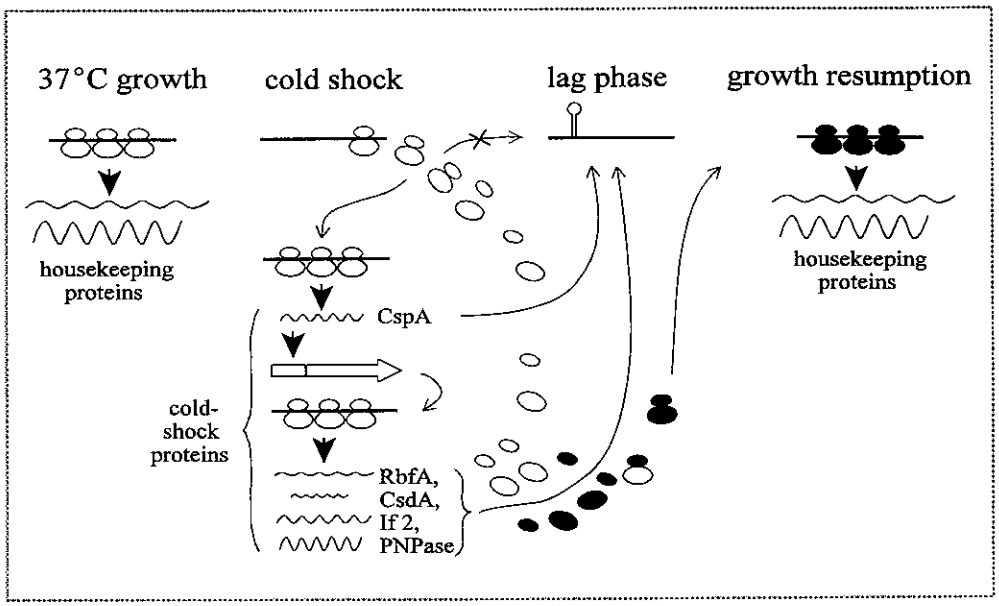

Figure 8.3 The Cold-Shock Ribosomal Adaptation model, as designed by Jones & Inouye and co-workers (described in Chapter 3), but now including a function for PNPase

8.6 PNPASE: REQUIRED FOR SUSTAINING DNA-SYNTHESIS IN THE COLD?

Recently, it was proposed that the true function of PNPase might be in coupling mRNA turnover to DNA synthesis in eubacteria [Danchin 1997]. This assumption was based on the results of an *in silico* analysis of a large number of annotated genomic sequences, which revealed the crucial position of nucleoside *di*-phosphates rather than *tri*-phosphates as precursors of deoxyribonucleotides.

The deduced essential role of PNPase in DNA-synthesis, especially under energy limiting conditions, can be understood when bearing in mind that:

- the precursors for deoxyribonucleotides are ribonucleoside *di*phosphates and not *tri*phosphates, and
- the synthesis pathways of the pyrimidine-bases of DNA, deoxycytidine- and deoxythymidine-triphosphate (dCTP and dTTP), are not via *de novo* synthesis but (almost) entirely via recycling of mRNA.

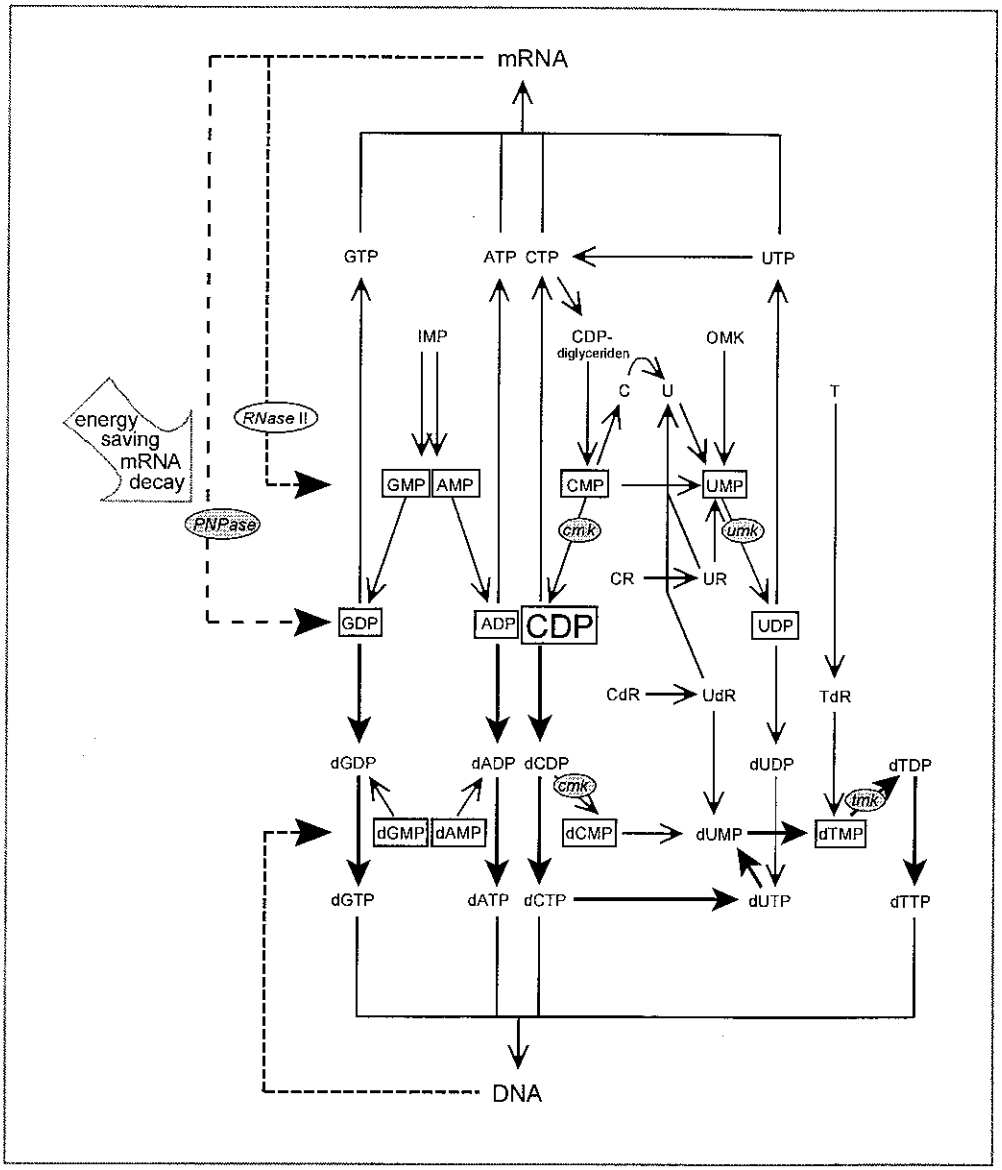

Figure 8.4 Pathways of synthesis (——) and breakdown (- - -) of DNA and RNA, including their nucleotide intermediates and several important enzymes. The products of mRNA and DNA decay are in boxes; enzymes are in ellipses. Deficiency in the enzymes shown in grey ellipses results in cold-sensitivity or loss of psychrotrophy.

A scheme of the major synthesis pathways of the nucleotide precursors for RNA and DNA is depicted in Figure 8.4, and shows that CDP, not UDP, is the ultimate precursor of dTTP. CDP, however, can only be produced in the cell by cycling reactions, i.e. by turnover of membrane phospholipids or by mRNA degradation.
The enzymatic mRNA-decay can proceed via either PNPase or RNaseII, but the production of nucleotidediphosphates via the RNaseII directed pathway requires an extra, energy-consuming(!), phosphorylation-step due to mRNA breakdown to the monophosphate level by this enzyme. This renders this route less efficient under energy-limiting conditions. In the light of this fact, it is striking that cold-sensitive RNaseII null mutants have never been described. This is in sharp contrast to the repeated isolation of cold-sensitive *pnp*-mutants, but also to the existence of several 'cold-sensitive' mutants that are deficient in other enzymes which are involved in DNA and RNA-synthesis, such as dTMP-kinase (*tmk*: Daws & Fuchs 1984), CMP-kinase (*cmk, or mmsA*: Fricke *et al.* 1995) and UMP-kinase (*umk, pyrH, or smbA*: Yamanaka *et al.* 1994; Serina *et al.* 1995). As can been seen in Figure 8.4, all of these enzymes are involved in conversion that are needed for the synthesis of dTTP. It has also been observed that overproduction of CMP-kinase can compensate for UMP-kinase deficiency [Yamanaka *et al.* 1992]. This does not only support the idea that CDP has a crucial position in the DNA-synthesis pathways, but also strongly suggests that *E. coli* switches to a PNPase-directed mRNA decay at reduced temperatures. In addition, the requirement of PNPase for competence development in *Bacillus subtilis* [Luttinger *et al.* 1996] has also been explained as an indication that the main function of PNPase is in providing the cell with CDP.
A leading role in for PNPase in the recycling of nucleotides is all the more likely because mRNA secondary structuring will increase at low temperatures, and PNPase degrades secondary-structured mRNA much more efficiently than RNaseII [Guarneros & Portier 1990]. Additional evidence for a major role for PNPase in coupling RNA-turnover to DNA-synthesis comes from our own observation that *Y. enterocolitica* apparently quits with redundant plasmid DNA at low temperatures. It is not unlikely that this phenomenon reflects a shortage in DNA-precursors under these conditions which cannot completely be prevented by an increased level and/or activity of PNPase.

For all of these reasons, I think we may conclude that PNPase has a key function in the cell with respect to sustaining DNA-synthesis at reduced temperatures.

Beyond the scope of this thesis...

From various studies which addressed the mechanisms that might effect stress induction in bacteria, it appeared that several other factors may be involved. Alternative RNA sigma factors, for example, might play a role via conferring promoter-specific transcription initiation on RNA polymerase [Kapatral & Minnich 1995; Sledjeski *et al.* 1996]. Furthermore, it was suggested that sigma-factor promoter specificities depend on the relative orientation of the -10 and -35 regions, and thus are linked with the level of DNA supercoiling [Wang & Syvanen 1992]. In fact, evidence is growing that DNA superhelix structure might function as a transcriptional sensor for a wide variety of environmental changes which alter cellular energetics, including temperature, in many bacterial species [Jones *et al.* 1992; Qoronfleh *et al.* 1992; Krispin & Allmansberger 1995; Grau *et al.* 1994], including Y. enterocolitica [Rohde *et al.* 1994]. However, experiments to investigate the role of alternative sigma factors or a change in DNA-twist in cold adaptation of *Yersinia enterocolitica* were beyond the scope of this thesis.

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Curriculum Vitae

The author, **Rosalina Lucia Jacoba Goverde** was born on the 20th of September in 1953 in Rotterdam as the eldest daughter of Jacoba Lucia van der Loo and Gerardus Antonius Goverde, who eventually became the parents of seven children.

After getting her **HBS-B certificate** at the local 'St.-Monfort college', in 1970, she studied **analytical and organic preparative chemistry** at the former laboratory school 'van't Hoff Instituut' in Rotterdam. She qualified as a **technician** in 1974, and subsequently was employed by: the plastic works 'Synthese' in Bergen op Zoom (1974-1975); the meat-products plant Gevato in Driebergen (1976-1977); the department of 'Science of Food of Animal Origin' (VVDO) at the State University of Utrecht (1977); and the Food Inspection Service in Utrecht (1977-1990). In these years, she was involved in plastic research, in chemical and microbiological quality-control of foods and related products, and in the development of analytical methods, especially for the detection of residues of veterinary medicines in foods of animal origin.

In 1983, she started to study **Biology**, taking the then part-time course at the University of Utrecht. In 1985, she cum laude passed her **preliminary-examinations**. In 1988, she specialized in immunology at the department of Infectious Diseases and Immunology from the Faculty of Veterinary Medicine, under supervision of Dr. W. van Eden. In a six-month study, and in collaboration with P. van Kooten, she worked on the generation and characterization of monoclonal antibodies against the antibiotic chloramphenicol. In 1989-1990, she qualified in microbiology, taking a course at the Faculty of Biology (Prof. Dr. W. Hoekstra, department of Molecular Microbiology) and working for eight months at the Department of Bacteriology from the National Institute of Public Health and the Environment (RIVM). At the RIVM, she worked on the development of a fast and easy, DNA-based method to detect and identify the human pathogenic bacterium Yersinia enterocolitica in foods and clinical samples. This work was done in cooperation with H. Brunings and W. Jansen, under the supervision of Dr. F.R. Mooi. In 1990, she graduated cum laude as a **molecular microbiologist**.

At the end 1990, she returned to the VVDO at the University of Utrecht, now as a **part-time PhD-student**, to start - under supervision of Prof. Dr. J.H.J. Huis in't Veld - the work on bacterial psychrotrophy which is described in this thesis. Halfway 1992, the breeding and growth-studies with Y. enterocolitica were interrupted for a couple months, to gave birth to a daughter and to indulge into the - even more fascinating - aspects of rearing a human being. After the work was resumed, she got the opportunity to join the team of Dr. Jan van Emden and 'his' molecular microbiologists, technicians and PhD-students, at the Department of

Molecular Microbiology (the present Laboratory for Research on Infective Diseases), at the RIVM. During 1994 and 1995, she worked in the inspiring company of these experts on bacterial pathogenesis, under supervision of Dr. F.R. Mooi. The **experimental work was rounded off in 1996**.

From 1996 to 1998, the author was involved in writing of Project proposals for financial support from the European Commission and from the Dutch Commodity Boards for Livestock, Meat and Eggs, for future research in the field of bacterial psychrotrophy.
Since 1999, she has entered again into the VVDO, to work at **detection methods** for other (mesophilic) food borne human pathogens, i.e. **Salmonella** species.

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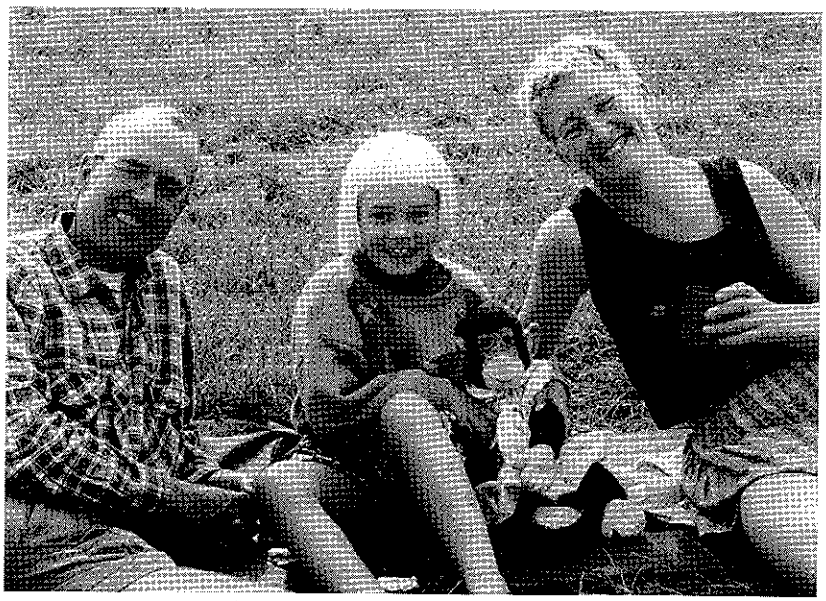

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Yersinia enterocolitica

een bacterie die kan groeien in de koelkast

Waar gaat dit proefschrift over?

Dit proefschrift gaat over *Yersinia enterocolitica*, een bacterie die bij de mens ernstige darmklachten kan veroorzaken, meestal na het eten van besmet voedsel. Net als bij vele andere ziekteverwekkers het geval is, zijn rauwe voedingsmiddelen van dierlijke oorsprong de belangrijkste bron van deze bacterie. Een groot verschil met de meeste andere voedselbesmetters is het feit dat *Y. enterocolitica* zich zelfs rond het vriespunt nog kan vermenigvuldigen. Dat betekent dat producten waarin kleine aantallen *Y. enterocolitica* voorkomen toch niet zonder risico in de koelkast bewaard kunnen worden. Snelle, eenvoudige en doeltreffende opsporingsmethoden zijn dus van groot belang om het risico voor de consument te verkleinen. In dit proefschrift wordt de ontwikkeling van zo'n methode beschreven.
Anderzijds is koeling een wijze van conserveren die weinig afbreuk doet aan de kwaliteit van levensmiddelen. Het is dus wenselijk om over een methode te beschikken waarmee we bacteriën kunnen verhinderen om te groeien bij lage temperatuur. Hiervoor is echter diepgaande kennis nodig over de manier waarop de bacterie zich aanpast aan de kou, en welke mechanismen hier een rol in spelen. Met het in dit proefschrift beschreven onderzoek proberen we een tipje van de sluier op te lichten.

Je bent wat je eet ...?

Leven en welzijn van de mens worden voor een groot gedeelte bepaald door zijn voeding: is er voldoende, is het lekker, is het gevarieerd genoeg, is het van goede kwaliteit? Geen wonder dat er wereldwijd veel aandacht wordt geschonken aan de kwaliteit van onze voeding. Niettemin zijn er legio verschillende ziekten bekend die worden overgebracht via ons eten of drinken: zelfs in de geïndustrialiseerde wereld wordt naar schatting jaarlijks 3-5% van de bevolking getroffen door een voedselinfectie. In 60 tot 70% van de gevallen zijn bacteriën de boosdoeners en meestal zijn dat soorten uit de geslachten Salmonella, Campylobacter en Shigella. Deze 'bekende' ziekteverwekkers zorgden met name in de jaren tachtig en negentig voor veel commotie, door hun betrokkenheid bij diverse grote en kleine uitbraken van voedselvergiftiging. Maar er zijn nog vele andere soorten bacteriën die aanleiding kunnen geven tot voedselinfecties en regelmatig treden 'nieuwe' soorten op de voorgrond. Denk maar aan de recente voedselvergiftigingen in Engeland, Japan en de Verenigde Staten met de toxine-producerende *Escherichia coli* O:157, een zeer venijnige variant van de normaliter onschuldige darmbacterie.

Door dergelijke ernstige uitbraken, waar vaak zeer veel mensen bij betrokken zijn, komen voedselinfecties als gezondheidsrisico weer eens in de schijnwerpers. Maar in feite vormen deze uitbraken slechts het topje van de ijsberg. Want bacteriële voedselinfecties worden vaak niet als zodanig herkend want de klachten zijn meestal niet zo ernstig : "ach, een beetje misselijk, wat buikpijn en diarree...". En meestal gaat dat binnen een paar dagen vanzelf weer over. De dokter komt er dan niet aan te pas, evenmin als laboratoriumonderzoek, en een diagnose "infectie met ..." wordt dan niet gesteld. Niettemin betreft het gros van de officieel geregistreerde gevallen van voedselinfectie op zichzelf staande gevallen, ondanks het feit dat er waarschijnlijk veel meer incidentele voedselinfecties voorkomen dan er gerapporteerd worden. En of het nu om uitbraken gaat of om op zichzelf staande gevallen, de aanleiding is veel gevallen dezelfde: het eten van rauwe of onvoldoende verhitte voedingsmiddelen, met name wanneer deze van dierlijke oorsprong zijn, zoals vlees, vis, melk, eieren en schaaldieren.

De bacterie *Yersinia enterocolitica*

Een 'nieuwe' ziekteverwekker

Een van de minder bekende ziekteverwekkers die men kan binnen krijgen via zijn voedsel is de bacterie *Yersinia enterocolitica* (Figuur 1.1). Hoofdstuk 1 geeft een overzicht van wat er zoal over deze bacteriesoort bekend is.

Het micro-organisme dat we nu kennen onder de naam *Y. enterocolitica* werd voor het eerst beschreven in 1934, na isolatie uit enkele slecht genezende huidzweren van een Amerikaanse boerenvrouw. In de daaropvolgende jaren werd een soortgelijke bacterie in Amerika af en toe

aangetroffen bij kinderen met ernstige ingewandsstoornissen. Op grond hiervan kreeg het micro-organisme voorlopig de naam *Bacterium enterocoliticum* alhoewel er, op grond van morfologische en biochemische eigenschappen, ook sprake was van een zekere verwantschap met bacteriën uit de *Pasteurella* groep, waarin men ook de beruchte pest-bacil had ondergebracht. Hierna raakte de bacterie enigszins in de vergetelheid. Daarin kwam verandering toen eind jaren vijftig identieke bacteriën werden geïsoleerd uit dieren die waren omgekomen tijdens grootschalige uitbraken van dierziekte in pelsdier-fokkerijen in Midden-Amerika. Ongeveer tezelfdertijd dook deze bacterie (intussen ook wel aangeduid met tot de verbeelding sprekende namen als *Pasteurella-X* en *Kiem-X*) ook in Europa op: niet alleen onder wilde en gekweekte knaagdieren, maar ook als ziekteverwekker bij de mens.
Daarmee werd de vooralsnog 'onbekende' bacterie onderwerp van koortsachtig onderzoek. In record tempo wist men de verspreiding ervan in kaart te brengen, en in 1964 werd er de naam *Yersinia enterocolitica* aan toegekend. De geslachtsnaam 'Yersinia' werd gekozen als eerbewijs aan Dr. Jean Emil Yersin (zijn portret is te zien op een postzegel uit Indochina, op pagina 7), de ontdekker van de nauw verwante pest-bacil (nu: *Yersinia pestis*), het achtervoegsel 'enterocolitica' verwijst naar het maag-darm kanaal, de plek waaruit de bacterie meestal werd geïsoleerd. Het ziektebeeld dat de bacterie kan veroorzaken werd daarna aangeduid met de term '**yersiniosis**'.

Verschillende typen

Intussen was echter al wel duidelijk geworden dat de bacterie niet alleen uit zieken kon worden geïsoleerd, maar ook uit water, uit de bodem, uit zieke en gezonde dieren, uit voedingsmiddelen etc. Bovendien bleek er sprake van een wereldwijde verspreiding (zie Tabel 1.1) . Al snel kon men vaststellen dat het - ondanks de onderlinge overeenkomsten - lang geen homogene groep bacteriën betrof: verschillende eigenschappen, zoals het vermogen om bepaalde chemische verbindingen om te zetten of de aanwezigheid van specifieke oppervlakte structuren (de zogenaamde O-antigenen), bleken behoorlijk te kunnen variëren naargelang de herkomst van het isolaat. Dit leidde tot een verdere onderverdeling: op grond van biochemische eigenschappen onderscheidt men tegenwoordig zes '**biotypen**', en al meer dan 60 '**O-serotypen**'. Het serotype onderscheid berust op het al of niet samenklonteren van de bacterie met antistoffen in het bloedserum van proefdieren die vooraf werden geïmmuniseerd met het betreffende O-antigeen. De meeste bio-/serotypen worden zelden of nooit in verband gebracht met ziekte bij de mens; slechts een drietal ervan (bio-/serotype (1B/O:8, 2/O:9 en 3/O:3) is verantwoordelijk voor 90% van de humane yersiniosis, terwijl zo'n tien andere typen sporadisch voorkomen bij mensen met yersiniosis-achtige klachten.

Voedsel-infecties

In de jaren zeventig en tachtig deden zich in Amerika en Japan verschillende grote en kleine uitbraken voor van yersiniosis. In lang niet alle gevallen werd de oorzaak opgehelderd, maar er waren wel steeds sterke aanwijzingen dat voedsel of drinkwater de bron van de besmetting

was geweest. In diezelfde tijd bleek dat de bacterie zich nu ook definitief had gevestigd in Noord-West Europa, getuige het veelvuldig voorkomen van yersiniosis in landen als België, Denemarken, Zweden, Noorwegen en Finland (zie Tabel 1.2 en 1.3). Ook hier stapelden de aanwijzingen zich op voor een verband tussen het optreden van de ziekte en het eten van besmet voedsel. De toename van het aantal ziektegevallen leidde tot een verdere intensivering van het onderzoek. Mede hierdoor is in de afgelopen drie decennia veel opgehelderd over deze veelzijdige bacterie. Men weet bijvoorbeeld goeddeels hoe *Y. enterocolitica* ziekte veroorzaakt (Figuur 1.2), welke factoren daarbij een rol spelen, zowel intern in de bacterie (Figuur 1.3) als extern bij de gastheer (Figuur 1.4), wat de bron van de ziekteverwekkende serotypen is, en hoe de overdracht naar de mens plaatsvindt (Figuur 1.6).

Varkens als bron

Varkens vormen de belangrijkste bron van humaan pathogene *Y. enterocolitica* stammen: de bacterie leeft als commensaal, dat wil zeggen zonder ziekte te veroorzaken, in hun maag/darm-kanaal. Tijdens de slacht kan echter het karkas besmet raken waardoor de bacterie in de voedselketen terecht komt. Rauw vlees is een uitstekend groei-medium voor allerlei micro-organismen, en *Y. enterocolitica* heeft daarbij als voordeel dat zij zich, in tegenstelling tot de meeste andere voedsel-pathogenen, ook bij lage temperatuur nog kan vermenigvuldigingen. Hierdoor kan een geringe besmetting van een stuk vlees op den duur grote gevolgen hebben: tijdens het bewaren in koelhuizen, een niet ongebruikelijke praktijk in de vleesverwerkende industrie, kan het aantal bacteriën schrikbarend toenemen. Dit kan ook gebeuren in de huishoudkoelkast en een onhygiënische keukenpraktijk kan bovendien aanleiding geven tot kruis-besmetting van andere voedingsmiddelen. Wie dergelijke besmette producten, rauw of na onvoldoende verhitting consumeert heeft dus grote kans op een Yersinia-infectie.

Vroegtijdige opsporing

In het afgelopen decennium is - met name in West-Europa - het aantal gevallen van yersiniosis aanmerkelijk teruggedrongen (Figuur 1.5), waarschijnlijk als gevolg van verbeteringen in de slachtpraktijk en gerichte publieksvoorlichting. Niettemin blijft het van belang om de aanwezigheid van de bacterie in voedingsmiddelen, of grondstoffen daarvoor, zo vroeg mogelijk op te sporen. En heeft er eenmaal een infectie plaatsgevonden dan is het zaak om zo snel vast te stellen of er inderdaad *Y. enterocolitica* in het spel is: enerzijds om de patiënt zo goed mogelijk te kunnen behandelen, anderzijds om verdere verspreiding te voorkomen. Om de opsporing van yersiniosis te verbeteren is in het kader van dit proefschrift een snelle en eenvoudige onderzoeksmethode ontwikkeld voor het opsporen en identificeren van deze ziekteverwekker. Hierbij is gebruik gemaakt van een techniek die ca. vijftien jaar geleden voor het eerst werd beschreven, de zogenaamde '**DNA-kolonie-hybridisatie**'. Hierbij worden kolonies van de bacterie, die gekweekt zijn op een agar-voedingsbodem,'herkend' doordat ze een kleurreactie geven met kunstmatig verkregen soort-specifiek erfelijk materiaal (DNA) (Figuur 4.7). Dit deel van het onderzoek is beschreven in hoofdstuk 4.

Het ontstaan van 'de koelketen'

Regelmatig is gesuggereerd dat er een verband bestaat tussen de opkomst van de ziekte yersiniosis en de grote vlucht die koeling heeft genomen als conserveringsmethode voor levensmiddelen. Om te bezien of daar inderdaad sprake van kan zijn geeft hoofdstuk 2 geeft een overzicht van het ontstaan de '**koelketen**' en de reikwijdte daarvan.

Sneeuw en ijs

De bederfwerende werking van koude was ook al in de oudheid bekend en werd ook zeker toegepast. Hiervan getuigen bijvoorbeeld de aanvoer van ijsgekoelde Noordzeevis op de markten in het oude Rome, en van in sneeuw verpakte pruimen en bamboescheuten naar het hof van de Chinese keizer. Toch kwam koeling als conserveringsmethode voor voedsel pas echt in zwang in de loop van de 19e eeuw. De eerste aanzet daartoe werd gedaan in Noord-Amerika en Scandinavië toen daar, rond 1825, de mechanisatie op gang kwam van de tot dan toe kleinschalige, handmatige winning van natuurijs. In enkele decennia vond een enorme schaalvergroting plaats in de handel in natuurijs, waardoor het mogelijk werd koeling ook in warmere streken op grote schaal toe te passen (Figuur 2.1). Als eerste werden speciale koelhuizen gebouwd voor vlees en vis, waarbij het aangevoerde ijs als koelelement werd gebruikt. Al snel breidde deze praktijk zich uit naar andere bedrijfstakken, zoals de fruithandel en de zuivelindustrie. Het transportbedrijf volgde en ook in de privé-sector deed de 'ijskast' deed zijn intrede, met name bij hotels, restaurants en welgestelde huishoudens (Figuur 2.2). Hiermee had de 'nieuwe' conserveermethode zich een definitieve plek verworven.

Technische vernieuwingen

Maar er kleefden ook bezwaren aan: het ijs kwam vaak rechtstreeks met het product in aanraking, hetgeen hoge eisen stelde aan de zuiverheid. En niet alleen mogelijke verontreinigingen maar ook het transport en de benodigde opslagcapaciteit zorgden voor problemen. Men zocht daarom al snel naar mogelijkheden ter vervanging van het natuurijs. Een eerste verbetering vond plaats in de tweede helft van de 19e eeuw, toen men erin slaagde kunstmatig ijs te produceren, waarna wijd en zijd ijsfabrieken werden opgezet. Maar de echte doorbrak kwam rond 1900 toen, in het kader van de industriële revolutie, allerlei nieuwe technieken werden ontwikkeld, waaronder het comprimeren van gassen en de opwekking van stroom. Hiermee deed de mechanische koeling zijn intrede, als eerste stap op weg naar het moderne koelbedrijf. In 1918 werd in Amerika de eerste huishoudkoelkast geïntroduceerd, en in minder dan 20 jaar wist deze de klassieke ijskast te verdringen in de helft van de Amerikaanse huishoudens. Onder invloed van allerlei in- en externe factoren, zoals de introductie van freon-gassen en de uitbreiding van het elektriciteitsnet, vond er in de dertiger jaren wereldwijd een enorme expansie plaats in de commerciële toepassing van koeling in de voedingsmiddelenindustrie en -handel; hiermee begon het stramien van een 'koelketen' zich af te tekenen. Na de Tweede Wereldoorlog zette deze ontwikkeling zich door in de rest van

de geïndustrialiseerde wereld. De handel in levensmiddelen werd meer en meer gecentraliseerd, en de supermarkt met koelvitrines deed zijn intrede. Met de inburgering van de huishoudkoelkast (Figuur 2.3) in de jaren zestig werd de laatste schakel gesloten in een keten van ononderbroken koeling van levensmiddelen, vanaf de productie tot aan de consumptie. Vandaag de dag is deze koelketen een onmisbare factor in de handel in voedingsmiddelen.

Yersiniosis door grootschalige koeling?

De inburgering van ijs- en koelkasten in het huishouden vond in Amerika dus plaats in de jaren dertig, in Europa en de rest van de geïndustrialiseerde wereld zo'n drie decennia later. Met een vertraging van ongeveer 10 jaar komt dit opmerkelijk goed overeen met de opkomst van yersiniosis in de betreffende gebieden: eerste isolaties in Amerika aan het eind van de jaren dertig, verspreiding in Europa vanaf eind zestiger jaren en fikse uitbraken in zowel Amerika als Japan vanaf begin jaren zeventig (Figuur 8.1). Overigens dateert uit dezelfde periode de opmars van voedselinfecties door *Listeria monocytogenes*, een andere bekende koude-tolerante bacterie (Tabel 2.2).

***Yersinia* infecties door bloedtransfusie**

Ook op een ander terrein is er een opvallende overeenkomst te bespeuren het langdurig koelen van een product enerzijds, en besmetting van de mens met *Y. enterocolitica* via dit product anderzijds, namelijk op het vlak van bloedtransfusies. In 1975 werd voor het eerst een geval van besmetting met *Y. enterocolitica* als gevolg van bloedtransfusie gerapporteerd (in Nederland). Sindsdien is er - wereldwijd - een duidelijke toename van dit soort bloedvergiftigingen opgetreden. Met een frequentie van naar schatting 1 maal op de 9 miljoen bloedtransfusies is het natuurlijk nog steeds een zeer zeldzame gebeurtenis, maar de sterftekans hierbij is zeer groot: inmiddels zijn er tientallen gevallen met dodelijke afloop geregistreerd. In veel gevallen bleek achteraf dat de donor van het besmette bloed enige tijd daarvoor een - al dan niet als yersiniosis herkende - episode van diarree had doorgemaakt.

Langdurige koeling van donorbloed

De rol die koeling in deze toename speelt ligt waarschijnlijk in een combinatie van twee factoren. Enerzijds is het sinds eind jaren zeventig steeds gebruikelijker geworden om donorbloed op splitsen in zijn componenten, namelijk in bloedcellen, bloedplaatjes, serum, en deze afzonderlijk, gekoeld, te bewaren. Anderzijds maakte deze opsplitsing een sterke verlenging van de opslagperiode mogelijk: bloed wordt tegenwoordig wel een maand bewaard alvorens het gebruikt wordt. Normaliter worden bacteriën die tijdens een infectie weten door te dringen tot de bloedbaan daar onschadelijk gemaakt door verschillende afweermechanismen. Een daarvan is het '**complement-systeem**', een reactie keten van allerlei stoffen die in opgeloste vorm in het serum voorkomen. De tweede belangrijke afweerreactie verzorgen bepaalde typen witte bloedcellen, die gespecialiseerd zijn in de afbraak van allerlei micro-organismen.

***Yersinia enterocolitica* kan ontsnappen aan de afweer...**

Y. enterocolitica is echter in staat gedeeltelijk aan beide afweersystemen te ontsnappen doordat altijd wel een paar bacteriën er in slagen zich te laten opnemen in andere typen bloedcellen, waarin ze niet kunnen worden afgebroken. Besmetting van het bloed is dan nauwelijks vast te stellen. Wanneer nu dit bloed gekoeld bewaard wordt zullen deze bacteriën na verloop van tijd weer vrijkomen, doordat de bloedcellen langzaam afsterven en uiteenvallen. Bij gescheiden opslag van bijvoorbeeld bloedplaatjes of rode bloedcellen blijven er altijd wel enkele witte bloedcellen aanwezig, maar de beide afweersystemen (de serumcomponenten en de gespecialiseerde witte bloedcellen) zijn dan praktisch gesproken afwezig. In dat geval hoeven er maar enkele bacteriën ontsnapt te zijn aan de eerste afweer om tot een probleem te leiden: ze kunnen zich dan immers onbelemmerde vermenigvuldigen bij afwezigheid van remmende factor. Maar ook als het besmette bloed niet van te voren wordt opgesplitst is langdurige koeling riskant omdat lage temperaturen de werkzaamheid van de beide afweersystemen ook nog eens sterk verminderen. Daar komt bij dat ook de rode bloedcellen na enige tijd uiteen beginnen te vallen, waardoor hun hemoglobine vrijkomt in de bloedvloeistof. Dit vormt dan een schier onuitputtelijke bron van opneembaar ijzer, een essentiële voedingsstof voor de bacterie. Concurrentie van andere bacteriën hoeft *Y. enterocolitica* in die situatie nauwelijks te duchten, omdat die vrijwel afwezig zijn, of niet in staat tot groei bij lage temperatuur.

***Yersinia enterocolitica* profiteert van 'de koelketen'**

Kortom: literatuurgegevens die zijn verzameld in het kader van dit proefschrift ondersteunen de veronderstelling dat er een oorzakelijk verband bestaat tussen de opkomst van yersiniosis enerzijds en het ontstaan van de 'koelketen' anderzijds (Figuur 8.1). Het langdurig gekoeld bewaren van in geringe mate besmette levensmiddelen en bloed producten verschaft de koude-tolerante bacterie *Y. enterocolitica* een ideale gelegenheid om zich ongehinderd te vermenigvuldigen en uit te groeien tot (levens)gevaarlijk hoge aantallen.

Bacteriën in de kou

Om te verhinderen dat een geringe aanvangsbesmetting leidt tot een enorme uitgroei tijdens gekoelde opslag, staan diverse methoden en technieken ter beschikking (Tabel 2.1). Te denken valt aan voorafgaande verhitting of doorstraling en het toevoegen van conserveermiddelen, maar het nadeel daarvan is dat ze de samenstelling en/of kwaliteit van het product nogal veranderen. Dat kan een reden zijn om van dergelijke conserveringsmethoden af te zien, maar dan zullen we er voor moeten zorgen dat de groei bij lage temperatuur wordt verhinderd of geremd. Dat is echter minder eenvoudig dan het wellicht lijkt. Om dit proces gericht te kunnen uitschakelen is diepgaande kennis nodig over het gedrag van de bacterie bij lage temperatuur:

hoe past hij zich aan de kou aan, en welke stofwisselingsprocessen spelen daarin een essentiële rol? De huidige kennis daarover is beschreven in hoofdstuk 3.

Bacteriële groei bij lage temperatuur

In het algemeen gesproken kunnen bacteriën groeien bij temperaturen tussen circa −10 en +110°C. Per soort liggen de grenzen echter veel nauwer, en op grond van de minimale, maximale en optimale groeitemperaturen kunnen vier groepen worden onderscheiden (Figuur 3.1). *Y. enterocolitica* behoort tot de groep van koude-tolerante ('**psychrotrofe**') bacteriën, soorten die zo worden genoemd omdat ze nog goed gedijen rond het vriespunt ook al hebben ze een voorkeur voor wat hogere temperaturen.

Veranderingen in eiwitten en vetten

Het onderzoek naar de manier waarop **psychrotrofen** zich aanpassen aan lagere temperaturen heeft de afgelopen decennia in het teken gestaan van de studie aan eiwitten en vetten, omdat deze macromoleculen, samen met het DNA (het erfelijk materiaal dat de samenstelling van de eiwitten bepaalt) en het RNA, de boodschappers tussen DNA en eiwitten, de belangrijkste bouwstoffen zijn van elke levende cel. Vetten vormen namelijk de 'huid' van de bacterie, de celmembraan, en eiwitten zorgen ervoor dat in de cel de benodigde levensprocessen plaatsvinden. Uit dit onderzoek is gebleken dat bijna alle bacteriën in staat te zijn om bij temperatuurverlaging hun vetten zodanig te veranderen dat de flexibiliteit van celmembraan gehandhaafd blijft. Koude-tolerante (en koude-minnende) bacteriën kunnen dit echter veel beter dan de soorten die niet bij lage temperatuur kunnen groeien. Ook wat de eiwitten betreft is men bepaalde temperatuurafhankelijke veranderingen op het spoor gekomen, namelijk de productie van zogenaamde Cold Shock Proteins (**CSPs**) en Cold Acclimation Proteins (**CAPs**) (Tabel 3.2 en 3.3). Zoals hun naam al aanduidt, worden de CSPs aangetroffen na een abrupte temperatuur-verlaging, in allerlei (!) bacteriën, terwijl de CAPs alleen worden gevonden in koude-tolerante en koude-minnende soorten. Men veronderstelt nu dat CAPs, en in zekere mate ook CSPs, een essentiële functie vervullen bij de gewenste aanpassing aan lage temperatuur (Figuur 3.2).

In het kader van dit proefschrift is onderzocht of de bovenbeschreven veranderingen in de samenstelling van vetten en eiwitten ook optreden in *Y. enterocolitica*. De resultaten van dat onderzoek zijn beschreven in hoofdstuk 5, en laten zien dat deze bacterie zijn vet-samenstelling inderdaad in sterke mate aanpast aan de omgevingstemperatuur (Figuur 5.3 en 5.4). Er werden echter geen grote veranderingen in de eiwitsamenstelling waargenomen. De gebruikte technieken waren overigens niet gevoelig genoeg om de productie van kleine hoeveelheden CAPs aan te tonen.

Erfelijke factoren voor aanpassing aan kou

Met het in hoofdstuk 5 beschreven structuuronderzoek werden in *Y. enterocolitica* wel enkele temperatuurafhankelijke wijzigingen in de eiwit- en vetsamenstelling waargenomen, maar dit leverde geen concrete aanwijzingen op over hoe deze veranderingen tot stand komen. In het vervolgonderzoek, beschreven in hoofdstuk 6, werd een poging ondernomen om dit proces verder te ontraadselen, door het opsporen van die delen in het DNA van de bacterie die onmisbaar zijn voor aanpassing aan kou.

Gemuteerde bacteriën: geen groei in de kou

Hiertoe werd gebruik gemaakt van een techniek die 'mutagenese' wordt genoemd. Hierbij brengt men in een groot aantal bacteriecellen willekeurige veranderingen aan in het DNA, waardoor een of meer eiwitten structureel veranderen of zelfs helemaal niet meer worden geproduceerd. Door gerichte selectie kan men dan die bacteriecellen opsporen die niet meer in staat zijn tot groei bij lage temperatuur (Figuur 6.3). In de betreffende cellen kan men vervolgens vaststellen welke delen van het DNA door de mutatie zijn beschadigd en welke eiwitten onklaar zijn gemaakt of wellicht ontbreken. Op deze wijze wisten we in *Y. enterocolitica* een DNA-fragment op te sporen waarvan de activiteit onmisbaar is voor het vermogen tot groei bij lage temperatuur (Figuur 6.1). Dit fragment, het '*pnp*-gen', is verantwoordelijk voor de aanmaak van PNPase, een eiwit dat van nature aanwezig is in allerlei micro-organismen. In de door ons gemuteerde bacteriën daarentegen, bleek geen PNPase te worden aangemaakt (Figuur 6.6). Tevens werd vastgesteld dat dit euvel verholpen kon worden door de mutant te 'repareren' met een extra *pnp*-gen (Figuur 6.2). We vonden bovendien dat de niet-gemuteerde *Y. enterocolitica* stam bij 5 °C beduidend meer PNPase aanmaakt dan bij 30 °C.

Het eiwit PNPase: onmisbaar in de kou

In het vervolgonderzoek, beschreven in hoofdstuk 7, is gezocht naar een verklaring voor de onmisbaarheid van PNPase voor groei bij lage temperatuur van *Y. enterocolitica* . Ook is gekeken hoe de bacterie er in slaagt om speciaal bij lage temperatuur meer van dit eiwit te produceren. Het lijkt er nu op dat specifieke structuren in het DNA, in de buurt van het *pnp*-gen, hiervoor verantwoordelijk zijn. Deze zijn namelijk niet te vinden bij de *pnp*-genen van bacterie-soorten die niet bij lage temperatuur kunnen groeien. Anderzijds werden dergelijke DNA-structuren wel weer gevonden in alle andere soorten van het geslacht *Yersinia,* hetgeen des te opmerkelijker is omdat die allemaal ook kunnen groeien bij lage temperatuur en dan extra PNPase aanmaken!

Kortom, er lijkt een duidelijk verband te zijn tussen de ruime beschikbaarheid van PNPase bij temperatuurverlaging en aanpassing aan de kou. PNPase vervult kennelijk een sleutelrol bij die aanpassing en die zou mogelijk kunnen liggen in het op gang houden van de DNA-synthese (Figuur 8.4).

Verder onderzoek moet uitwijzen of deze veronderstelling juist is en hoe we van die wetenschap gebruik kunnen maken om groei van bacteriën bij lage temperatuur te verhinderen.

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