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SEQUENCE VARIABILITY OF VIRULENCE GENES AND STRESS RESPONSES IN *YERSINIA PSEUDOTUBERCULOSIS*

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ACADEMIC DISSERTATION

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ABSTRACT

Yersinia pseudotuberculosis infections derive from ingestion of contaminated food or water. Typical symptoms of yersiniosis are fever and abdominal pain resulting from mesenteric lymphadenitis, and immunological sequelae are possible. The pathogen has recently caused several epidemics in Finland through fresh produce. However, the slow growth rate and poor competition of *Y. pseudotuberculosis* make its detection and isolation demanding. Polymerase chain reaction with primers targeted to virulence genes *inv*, *virF*, and *yadA* is thus often used in detection, although the sequence variability of the virulence genes is unknown. To study genetic variability of the virulence genes, *inv*, *virF*, and *yadA* of 18 *Y. pseudotuberculosis* strains, and two *Yersinia similis* strains originating from 12 different countries were sequenced. The greatest sequence variability was detected in *yadA*, while the variability of *inv* and *virF* was limited. The observed variability in *yadA* may hinder detection using PCR and also impact functional properties of YadA. Furthermore, the commonly used primers targeted to *inv* can, in addition to *Y. pseudotuberculosis*, detect *Y. similis*.

Y. pseudotuberculosis tolerates well low temperature and other stressful conditions in the environment and in the food chain. However, information on the stress tolerance mechanisms used by this pathogen is limited. Here, the roles of two-component systems (TCSs), alternative sigma factor σ^E, and RNA helicase CsdA of *Y. pseudotuberculosis* IP32953 under stress conditions were studied. The relative expression levels of 54 genes encoding putative TCSs in *Y. pseudotuberculosis* IP32953 were determined at 3°C and at the optimum growth temperature of 28°C. The relative expression levels of most of the genes were higher at 3°C than at 28°C, and TCS CheA/CheY encoding genes *cheA* and *cheY* had the highest relative expression levels at 3°C. Mutational analysis demonstrated the demand for *cheA* for optimal growth at 3°C. In addition, both *cheA* and *cheY* were required for motility. Increased expression of several TCS encoding genes demonstrate that probably in *Y. pseudotuberculosis* many TCSs play a role in adaptation to low temperatures. In addition, motility seems to be associated with cold tolerance.

The role of alternative sigma factor σ^E under stress conditions was studied by determining relative expression levels of *rpoE* encoding σ^E and using mutational analysis. Expression of *rpoE* was induced under low and high temperatures, acid and alkaline conditions, and osmotic and ethanol stress. Mutation of *rpoE* impaired or abolished growth at pH 5.0, at 3°C, at 37°C, at 42°C, and at 3% ethanol, demonstrating that functional σ^E is essential under several stress conditions in *Y. pseudotuberculosis* IP32953. In addition, the *rpoE* mutant had a higher minimum and a lower maximum growth temperature than the wild-type strain. Thus, in this pathogen, σ^E has a significant role in stress tolerance, and it contributes to survival during food processing and storage.

The function of a cold-induced RNA helicase CsdA has been unknown in *Y. pseudotuberculosis*. Investigation of the role of CsdA at 3°C by mutagenesis revealed that CsdA is essential for growth at low temperatures. At the optimum growth temperature of 28°C, no growth defect was seen. Also the minimum growth temperature of one of the mutants was significantly higher than that of the wild-type strain. Thus, CsdA enables the growth of *Y. pseudotuberculosis* in the food chain by allowing continuous growth at low

temperatures. The results demonstrate that the foodborne pathogen *Y. pseudotuberculosis* counters environmental stress by using TCSs and alternative sigma factor, and by synthesizing cold-induced proteins.

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Contents

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by their Roman numerals:

I Palonen, E., Kangas, S., Somervuo, P., Lindström, M., Fredriksson-Ahomaa, M., Skurnik, M., and Korkeala, H. (2013) Sequencing of virulence genes shows limited genetic variability in *Yersinia pseudotuberculosis*. Foodborne Pathog Dis 10: 21-27.

II Palonen, E., Lindström, M., Karttunen, R., Somervuo, P., and Korkeala, H. (2011) Expression of signal transduction system encoding genes of *Yersinia pseudotuberculosis* IP32953 at 28°C and 3°C. PLoS ONE 6: e25063. doi:10.1371/journal.pone.0025063.

III Palonen, E., Lindström, M., Somervuo, P., and Korkeala, H. (2013) Alternative sigma factor σ^E has an important role in stress tolerance of *Yersinia pseudotuberculosis* IP32953. Appl Environ Microbiol 79: 5970-5977.

IV Palonen, E., Lindström, M., Somervuo, P., Johansson, P., Björkroth, J., and Korkeala, H. (2012) Requirement for RNA helicase CsdA for growth of *Yersinia pseudotuberculosis* IP32953 at low temperatures. Appl Environ Microbiol 78: 1298-1301.

ABBREVIATIONS

Ail	attachment invasion locus
bp	base pair
cDNA	complementary DNA
CIN	cefsulodin-irgasan-novobiocin
Cip	cold-induced protein
C_q	quantification cycle
CSD	cold-shock domain
Csp	cold-shock protein
DFHEH	Department of Food Hygiene and Environmental Health
HK	histidine kinase
HPI	high-pathogenicity island
Hpt	histidine phosphotransferase
Inv	invasin
LB	Luria Bertani
LPS	lipopolysaccharide
M cell	microfold cell
OD_{600}	optical density at 600 nm
PCA	plate count agar
PCR	polymerase chain reaction
PNPase	polynucleotide phosphorylase
pYV	plasmid for *Yersinia* virulence
Rnase	ribonuclease
RovA	regulator of virulence
RR	response regulator
RT-qPCR	quantitative real-time reverse transcription polymerase chain reaction
SMAC	MacConkey agar supplemented with sorbitol
sRNA	small RNA
Syc	specific Yop chaperone
TCS	two-component signal transduction system
TSA	tryptic soy agar
UTR	untranslated region
VirF	transcriptional activator of the *Yersinia* virulence regulon
YadA	*Yersinia* adhesin
Yop	*Yersinia* outer membrane protein
YPM	*Y. pseudotuberculosis*-derived mitogen

1 INTRODUCTION

Yersinia pseudotuberculosis was first isolated in 1883 from guinea pigs that had been inoculated with contents of subcutaneous tubercles of a human patient (Malassez & Vignal, 1884, Mollaret, 1995). Initially, the pathogen was isolated only from rodents and birds (Mollaret, 1995). Human systemic infection and mesenteric adenitis caused by *Y. pseudotuberculosis* were reported in 1909 and 1910, respectively (Albrecht, 1910, Saisawa, 1913). In 1953, a form of mesenteric lymphadenitis affecting mainly children and youths was described (Masshoff & Dölle, 1953), and in 1954, *Y. pseudotuberculosis* isolated from mesenteric lymph nodes was shown to be the cause of the disease (Knapp & Masshoff, 1954, Knapp, 1954). Mesenteric adenitis caused by *Y. pseudotuberculosis* was subsequently reported in several European countries, and *Y. pseudotuberculosis* septicemia as well as immunological sequelae, including erythema nodosum, scarlatiniform rash, and reactive arthritis, were also reported (Mollaret, 1995).

The first *Y. pseudotuberculosis* epidemic was described in 1984 in Finland (Tertti *et al.*, 1984). The source of *Y. pseudotuberculosis* was not found, but several patients had been growing, selling, or eating vegetables (Tertti *et al.*, 1984). In 1998, in a large Canadian *Y. pseudotuberculosis* outbreak milk was suspected to be the source of infection (Nowgesic *et al.*, 1999). However, not until 2004 was *Y. pseudotuberculosis* confirmed to be a foodborne pathogen in a Finnish outbreak investigation (Nuorti *et al.*, 2004, Tauxe, 2004). Since then, several epidemics through contaminated fresh produce (Jalava *et al.*, 2004, Jalava *et al.*, 2006, Kangas *et al.*, 2008, Rimhanen-Finne *et al.*, 2009) and an epidemic through unpasteurized milk (http://www.promedmail.org/) have been reported in Finland.

Food, environment, and patient samples contain abundant background flora, making detection of *Y. pseudotuberculosis* slow and uncertain. Polymerase chain reaction (PCR) with primers targeted to virulence genes of *Y. pseudotuberculosis* is thus often used for detection and identification of this pathogen (Fredriksson-Ahomaa & Korkeala, 2003, Fredriksson-Ahomaa *et al.*, 2010, Skurnik *et al.*, 2009). However, the variability of the virulence genes in different *Y. pseudotuberculosis* strains and serotypes remains unknown.

Refrigeration is the most common preservation method used in the modern food chain. *Y. pseudotuberculosis* tolerates well low temperatures as well as other stressful conditions in the environment and food chain (Fredriksson-Ahomaa *et al.*, 2010, Fukushima *et al.*, 1989, Jalava *et al.*, 2006, Palonen *et al.*, 2010, Rimhanen-Finne *et al.*, 2009). However, studies of *Y. pseudotuberculosis* and stress factors encountered in the food chain are scarce, and thus, the mechanisms allowing stress tolerance of *Y. pseudotuberculosis* are largely unknown.

The first aim of this study was to map the genetic variability of virulence genes used for detection and identification of *Y. pseudotuberculosis*. To detect *Y. pseudotuberculosis* correctly by PCR, the sequence variability of the chosen genes should be low. The second aim was to investigate the role of two-component systems, sigma factor E, and RNA helicase CsdA in stress tolerance of *Y. pseudotuberculosis*. An understanding of how bacteria tolerate stress is necessary to be able to control pathogens in the food chain.

2 REVIEW OF THE LITERATURE

2.1 Genus *Yersinia*

The gram-negative bacterium *Yersinia pseudotuberculosis* belongs to the genus *Yersinia* of the family *Enterobacteriaceae* (Bottone *et al.*, 2005). Other species of the genus are *Y. aldovae, Y. aleksiciae, Y. bercovieri, Y. enterocolitica, Y. entomophaga, Y. frederiksenii, Y. intermedia, Y. kristensenii, Y. massiliensis, Y. mollaretii, Y. nurmii, Y. pekkanenii, Y. pestis, Y. rohdei, Y. ruckeri, Y. similis*, and *Y. wautersii* (Bottone *et al.*, 2005, Hurst *et al.*, 2011, Merhej *et al.*, 2008, Murros-Kontiainen *et al.*, 2011a, Murros-Kontiainen *et al.*, 2011b, Savin *et al.*, 2014, Sprague *et al.*, 2008, Sprague & Neubauer, 2005). *Y. entomophaga* and *Y. nurmii* are phylogenically very close to each other (Johanna Björkroth, personal communication). *Yersinia* are facultatively anaerobic, oxidase-negative, and catalase-positive, and they do not form spores (Bottone *et al.*, 2005). The optimum growth temperature of *Yersinia* is 28–29°C and their growth range is 4–42°C (Bottone *et al.*, 2005), but *Y. enterocolitica* and *Y. pseudotuberculosis* can grow at temperatures near 0°C (Bergann *et al.*, 1995, Bottone *et al.*, 2005, Fredriksson-Ahomaa *et al.*, 2010, Walker *et al.*, 1990). Three species are pathogenic to humans: *Y. pseudotuberculosis*, *Y. enterocolitica*, and *Y. pestis* (Bottone *et al.*, 2005). *Y. pseudotuberculosis* and *Y. enterocolitica* are enteropathogenic, while *Y. pestis* is the causative agent of plague (Carniel *et al.*, 2006). *Y. pseudotuberculosis* and *Y. pestis* are genetically highly similar and *Y. pestis* probably emerged from *Y. pseudotuberculosis* some 1500-20 000 years ago (Achtman *et al.*, 1999).

Y. pseudotuberculosis can be divided into 21 serotypes (Carniel *et al.*, 2006). Serotypes O:1a and O:1b are the most common ones isolated from patients in Europe, Australasia, and North America, and serotypes O:4b and O:5b from patients in East Asia (Carniel *et al.*, 2006). In South Korea, strains belonging to serotype O:15 are often found in human patients (De Castro *et al.*, 2009, Fukushima *et al.*, 2001, Laukkanen-Ninios *et al.*, 2011).

The first *Y. pseudotuberculosis* strain was sequenced in 2004 (Chain *et al.*, 2004). The genome of the sequenced strain IP32953 consists of a chromosome and two plasmids. The chromosome of the strain IP32953 is 4.7 Mbp long and contains 3974 coding sequences (Chain *et al.*, 2004).

2.2 *Yersinia pseudotuberculosis* infections

In 2010, *Yersinia* was the third most commonly reported zoonosis in the European Union (European Food Safety Authority, 2012). Most *Yersinia* cases were caused by *Y. enterocolitica* (European Food Safety Authority, 2012). However, in Finland *Y. pseudotuberculosis* has caused on average 16% (variation 5–32%) of confirmed *Yersinia* cases in the last decade (Jaakola *et al.*, 2012), and the incidence of *Y. pseudotuberculosis*

infections between 1995 and 2006 varied between 0.6 and 4.8 (average 1.9/100 000 population) (Fredriksson-Ahomaa *et al.*, 2010). *Y. enterocolitica* infections are usually sporadic, while *Y. pseudotuberculosis* has caused several outbreaks, especially in Finland and in Japan (Jalava *et al.*, 2004, Jalava *et al.*, 2006, Kangas *et al.*, 2008, Nuorti *et al.*, 2004, Rimhanen-Finne *et al.*, 2009, Tsubokura *et al.*, 1989).

2.2.1 Sources of *Yersinia pseudotuberculosis*

Y. pseudotuberculosis outbreaks have resulted from consumption of vegetables (Jalava *et al.*, 2004, Jalava *et al.*, 2006, Kangas *et al.*, 2008, Nuorti *et al.*, 2004, Rimhanen-Finne *et al.*, 2009) or other foodstuff (Tsubokura *et al.*, 1989), water (Tsubokura *et al.*, 1989), or unpasteurized milk (http://www.promedmail.org/) contaminated with the bacterium. Sporadic infections have arisen from ingestion of *Y. pseudotuberculosis* via water (Fukushima *et al.*, 1988, Fukushima *et al.*, 1989). *Y. pseudotuberculosis* has been isolated from wild animals such as rats (Fukushima *et al.*, 1988, Kageyama *et al.*, 2002, Zheng *et al.*, 1995), mice (Buhles *et al.*, 1981, Fukushima *et al.*, 1990), shrew (Kangas *et al.*, 2008), raccoon dogs (Fukushima & Gomyoda, 1991), deer (Fukushima & Gomyoda, 1991, Jerrett *et al.*, 1990), hares (Fukushima & Gomyoda, 1991), marten (Fukushima & Gomyoda, 1991), birds (Cork *et al.*, 1995, Fukushima & Gomyoda, 1991), and wild boars (Fredriksson-Ahomaa *et al.*, 2009).

Domestic animals may also harbour *Y. pseudotuberculosis*. Swine are potential reservoirs for *Y. pseudotuberculosis* infections (Laukkanen *et al.*, 2008, Laukkanen *et al.*, 2003, Niskanen *et al.*, 2008, Niskanen *et al.*, 2002, Okwori *et al.*, 2009, Ortiz Martínez *et al.*, 2010). In addition, the bacterium has been isolated from sheep (Okwori *et al.*, 2009, Slee & Skilbeck, 1992), goats (Lãtnada *et al.*, 2005), cattle (Slee *et al.*, 1988), rabbits (Zheng *et al.*, 1995), cats (Fukushima *et al.*, 1985, Fukushima *et al.*, 1989), and dogs (Fukushima *et al.*, 1985). Most human and animal yersiniosis cases are detected during winter, spring, or early summer (Carniel *et al.*, 2006, Fukushima *et al.*, 1985, Fukushima *et al.*, 1990, Jerrett *et al.*, 1990, Slee & Skilbeck, 1992, Slee *et al.*, 1988). Consumption of vegetables from the previous crop year has led to epidemics in the spring in Finland (Jalava *et al.*, 2006, Kangas *et al.*, 2008).

2.2.2 Symptoms

Infection with *Y. pseudotuberculosis* causes acute abdominal pain, mesenteric lymphadenitis, fever, and diarrhoea (Carniel *et al.*, 2006, Wren, 2003). Immunological sequelae, such as reactive arthritis and erythema nodosum, may follow infection (Fredriksson-Ahomaa *et al.*, 2010, Jalava *et al.*, 2006). In immunocompromised persons or persons with underlying diseases, systemic infections are possible (Carniel *et al.*, 2006).

2.3 Pathogenesis and virulence factors

Infection with *Y. pseudotuberculosis* follows ingestion of contaminated food or water (Fukushima *et al.*, 1988, Fukushima *et al.*, 1989, Jalava *et al.*, 2004, Jalava *et al.*, 2006, Kangas *et al.*, 2008, Nuorti *et al.*, 2004, Rimhanen-Finne *et al.*, 2009, Tsubokura *et al.*, 1989). In the terminal ileum, bacteria are internalized by microfold (M) cells. From the M cells, *Y. pseudotuberculosis* terminates in lymphoid follicles under the intestinal epithelium (Leo & Skurnik, 2011). *Yersinia* can spread from the lymphoid follicles via lymphatics and blood vessels to the mesenteric lymph nodes, spleen, liver, lungs, and peripheral lymph nodes (Carniel *et al.*, 2006). Previously healthy persons usually have localized *Yersinia* infections in the gut wall or regional lymph nodes, while immunocompromised persons or persons with underlying diseases can have systemic infections (Carniel *et al.*, 2006).

The 70-kb virulence plasmid pYV (plasmid for *Yersinia* virulence) is essential for virulence and is present in all *Y. pseudotuberculosis* strains that are pathogenic (Carniel *et al.*, 2006, Gemski *et al.*, 1980). Other important virulence factors are the adhesins invasin (Inv) and attachment invasion locus (Ail). In addition, a high-pathogenicity island (HPI) and/or superantigenic toxins (*Y. pseudotuberculosis*-derived mitogen, YPM) can be found in some *Y. pseudotuberculosis* strains (Carniel *et al.*, 2006). *Y. pseudotuberculosis* can be divided into genetic groups based on the presence of HPI and YPM (Table 1) (Fukushima *et al.*, 2001).

Table 1. Genetic groups of *Yersinia pseudotuberculosis* (Fukushima *et al.*, 2001).

Genetic group	Pathogenicity	HPI[1]	YPM[2] subtype	Serotype
1	+	+	YPMa	O:1b, 3, 5a, 5b, 15[3]
2	+	+	-	O:1a, 1b, 3, 5b, 13, 14
3	+	-	YPMa	O:1b, 1c, 2a, 2b, 2c, 3, 4a, 4b, 5a, 5b, 6, 7, 10, 15[3]
4	-	-	YPMb	O:1b, 5a, 5b, 6, 7, 9, 10, 11, 12
5	Low	Truncated	YPMc	O:3
6	+	-	-	O:1b, 2a, 2b, 2c, 3, 4a, 4b, 5a, 5b, 6, 7, 10, 11, 13, 15[3]

[1]HPI, high-pathogenicity island
[2]YPM, superantigenic toxin
[3]Serotype 15 included in the group in Carniel *et al.* (2006)

2.3.1 High-pathogenicity island

To disseminate in the host, a bacterium must have the ability to acquire ferric ions in the iron-restricted environment of the host (Carniel, 2001). High-pathogenicity *Yersinia* strains have a well-conserved HPI that encodes a siderophore yersiniabactin used for ferric ion uptake (Carniel, 2001, Carniel *et al.*, 1987, Carniel *et al.*, 1989, de Almeida *et al.*, 1993). Generally, HPI-carrying *Yersinia* strains are capable of systemic dissemination in the host, while *Yersinia* strains not carrying HPI are less pathogenic and cause localized infections (Carniel, 1999). Low-pathogenicity *Yersinia* strains usually cause systemic infections only in iron-overloaded patients (Carniel *et al.*, 2006). In addition to *Yersinia*, HPI is also present in other *Enterobacteriaceae* species (Carniel, 2001).

Several highly virulent *Y. pseudotuberculosis* O:1 and O:3 strains do not encode yersiniabactin, which has led to the suggestion of the existence of alternative siderophores (Rakin *et al.*, 2012). Two gene clusters, existing in all of the sequenced *Y. pseudotuberculosis* and *Y. pestis* strains, encode pseudochelin and yersiniachelin, which may function as siderophores (Rakin *et al.*, 2012).

2.3.2 Invasin

Expression of the chromosomally encoded virulence factor adhesin Inv (Isberg *et al.*, 1987) is thermoregulated. Expression of Inv is highest at moderate temperatures (25-28°C) during the late stationary phase (Isberg *et al.*, 1988, Nagel *et al.*, 2001). At 25°C during the exponential growth phase or at 37°C during the stationary growth phase, expression of Inv is low, and at 37°C during the exponential growth phase expression of it is virtually undetectable (Nagel *et al.*, 2001). Transcriptional regulator designated RovA (regulator of virulence) (Nagel *et al.*, 2001, Revell & Miller, 2000) induces transcription of inv at moderate temperatures (Nagel *et al.*, 2001). Both RovA and Inv are required for efficient invasion of *Y. pseudotuberculosis* to mammalian cells (Marra & Isberg, 1997, Nagel *et al.*, 2001). At 37°C RovA is degraded proteolytically (Quade *et al.*, 2012). Low expression of Inv and degradation of RovA at mammalian body temperature contradict the role of Inv as a virulence factor. However, other environmental signals can induce Inv expression in the host (Nagel *et al.*, 2001). At 37°C optimal *inv* expression requires acidic conditions in *Y. enterocolitica* (Pepe *et al.*, 1994), but in *Y. pseudotuberculosis*, acidic pH does not induce *inv* or *rovA* expression at 37°C (Nagel *et al.*, 2001). In *Y. pseudotuberculosis*, nutrient-rich environment and physiological osmolarity induce *rovA* and *inv* expression (Nagel *et al.*, 2001), while in *Y. enterocolitica*, nutrient content of growth medium does not affect *inv* expression and physiological osmolarity decreases *inv* expression (Pepe *et al.*, 1994).

Adhesin Inv has an important role at the beginning of *Yersinia* infection. In the terminal ileum, Inv binds to the β1 integrins on the apical surfaces of the M cells, leading to internalization of bacteria by the M cells (Leo & Skurnik, 2011). Invasin causes an inflammatory response in epithelial cells, which may help *Yersinia* to disseminate in the

host (Grassl *et al.*, 2003). Primers targeted to *inv* are commonly used to detect *Y. pseudotuberculosis* (Fredriksson-Ahomaa *et al.*, 2010, Skurnik *et al.*, 2009).

2.3.3 Attachment invasion locus

Chromosomally encoded adhesin Ail is expressed at 37°C (Leo & Skurnik, 2011). Ail of *Y. enterocolitica* adheres to epithelial cells and plays a role in invasion of some cell types such as human laryngeal epithelium in tissue culture (Leo & Skurnik, 2011, Miller & Falkow, 1988). In an earlier report, Ail of *Y. pseudotuberculosis* did not mediate cellular adhesion or uptake to eukaryotic cells when investigated with an *Escherichia coli* expression system (Yang *et al.*, 1996). Later, this defect was found to be due to substitution of threonine at position 7 with isoleucine in the Ail of the *Y. pseudotuberculosis* strain used (Tsang *et al.*, 2013). When the substitution is not present, Ail of *Y. pseudotuberculosis* mediates both cellular adhesion and uptake in the *E. coli* expression system. However, Ail expressed in *Y. pseudotuberculosis* is unable to mediate cell adhesion or invasion in vitro probably due to the long lipopolysaccharide (LPS) of *Y. pseudotuberculosis* (Tsang *et al.*, 2013). It has been suggested that in *Y. enterocolitica* significance of Ail in serum resistance is small because O-antigenic chains of the LPS often mask this small outer membrane protein (Biedzka-Sarek *et al.*, 2005, Wachtel & Miller, 1995). However, in *Y. pseudotuberculosis* Ail mediates serum resistance even in the presence of the full-length LPS (Ho *et al.*, 2012a, Ho *et al.*, 2012b, Paczosa *et al.*, 2014, Yang *et al.*, 1996). In addition, Ail has a role in the colonization and growth of *Y. pseudotuberculosis* in the lungs (Paczosa *et al.*, 2014).

2.3.4 *Yersinia pseudotuberculosis*-derived mitogen

Part of the *Y. pseudotuberculosis* strains produce superantigen YPM in the host (Abe *et al.*, 1993, Miyoshi-Akiyama *et al.*, 1993, Uchiyama *et al.*, 1993) (Table 1). Superantigens induce host cells to secrete large amounts of cytokines that can launch or worsen autoimmune diseases (Carniel *et al.*, 2006). There are three variants of YPM: YPMa, YPMb, and YPMc. At the amino acid level, YPMb differs from YPMa by 17%, and YPMc has one amino acid substitution compared with YPMa (Carniel *et al.*, 2006). Genes encoding YPMa and YPMc have probably evolved from *ypmB*, which is supposed to be an ancestral *ypm* gene (Carnoy *et al.*, 2002). Production of the YPM increases the virulence of *Yersinia* (Carnoy *et al.*, 2000), and based on the presence of *ypmB* in non-pathogenic *Y. pseudotuberculosis* strains (Table 1), genetic drift from *ypmB* to *ypmA* and *ypmC* has been suggested (Carnoy *et al.*, 2002).

2.3.5 Plasmid for *Yersinia* virulence

Genes encoding for *Yersinia* outer membrane proteins (Yops) and *Yersinia* adhesin (YadA) are located in the pYV (Cornelis & Wolf-Watz, 1997). Yops are secreted proteins and essential for virulence. Some of them, such as YopE, YopH, YpkA, and YopM, function as intracellular effectors in eukaryotic cells and others, such as YopD and YopB, are involved in the translocation of other Yops in the eukaryotic cells (Cornelis & Wolf-Watz, 1997). When *Yersinia* adhere to eukaryotic cells, Yops are secreted into eukaryotic cells by a pYV-encoded type III secretion apparatus Ysc, which forms a hollow channel for Yops from the bacterial cytoplasm to the eukaryotic cytoplasm (Bergman *et al.*, 1994, Cornelis, 2006, Michiels *et al.*, 1991, Rosqvist *et al.*, 1994, Rosqvist *et al.*, 1995). Specific Yop chaperones (Sycs) assist in the secretion of Yops (Cornelis & Wolf-Watz, 1997, Cornelis, 2006). Adhesins Inv, Ail, and YadA enhance Yop translocation into host cells (Maldonado-Arocho *et al.*, 2013, Mejía *et al.*, 2008) and play a role in serum resistance in *Y. pseudotuberculosis* (Maldonado-Arocho *et al.*, 2013). When bacteria are in close contact with eukaryotic cells, *yop* genes are transcribed (Pettersson *et al.*, 1996). Also a pYV-encoded transcriptional activator of the *Yersinia* virulence regulon (VirF) positively controls the transcription of *yop*, *ysc*, and *yadA* (Cornelis *et al.*, 1989, Heroven *et al.*, 2012a, Michiels *et al.*, 1991). In *Y. pseudotuberculosis*, transcription of *virF* is repressed by a small nucleoid-structuring protein YmoA (*Yersinia* modulator A) (Böhme *et al.*, 2012, Cornelis *et al.*, 1991, Heroven *et al.*, 2012a). At 37°C proteases degrade YmoA and transcription of *virF* is induced (Heroven *et al.*, 2012a). In addition, at 25°C *virF* mRNA of *Y. pseudotuberculosis* contains a hairpin structure that blocks the ribosomal binding site (Böhme *et al.*, 2012). At 37°C the hairpin structure melts and *virF* mRNA is translated, resulting in induction of pYV-encoded virulence factors. Compared with the wild-type strain, stabilization of the hairpin structure results in attenuated virulence, while destabilization leads to either similar or attenuated virulence. Thus, an exact amount of VirF is needed for ideal pathogenicity of *Y. pseudotuberculosis* (Böhme *et al.*, 2012).

After getting through the intestinal epithelium, YadA (Yang & Isberg, 1993) is the principal adhesin that is used by *Yersinia* (Leo & Skurnik, 2011). Expression of pYV-encoded *yadA* is highest at 37°C in the exponential growth phase in a minimal medium (Eitel & Dersch, 2002). The structure of YadA resembles a lollipop projecting from the bacterial cell surface (Hoiczyk *et al.*, 2000). It binds collagen, fibronectin and laminin, intestinal mucus, and many cell types such as epithelial cells, macrophages (Leo & Skurnik, 2011), and neutrophils (Paczosa *et al.*, 2014). In fact, in *Y. pseudotuberculosis*, YadA can be used instead of Inv in the invasion of epithelial cells (Bliska *et al.*, 1993, Eitel & Dersch, 2002, Yang & Isberg, 1993) because the N-terminal amino acid sequence of the YadA protein of *Y. pseudotuberculosis* can mediate uptake to epithelial cells (Heise & Dersch, 2006). YadA elicits an inflammatory response in host cells (Eitel *et al.*, 2005). Resistance to complement, phagocytosis, and antimicrobial peptides are also governed by YadA (Ho *et al.*, 2012a, Leo & Skurnik, 2011, Paczosa *et al.*, 2014). YadA is a haemagglutinin and autoagglutinin (Heise & Dersch, 2006) and contributes to type III secretion (Leo & Skurnik, 2011, Paczosa *et al.*, 2014). In *Y. pseudotuberculosis*, YadA

together with chromosomally encoded Ail are required for persistence in the lungs and dissemination to the spleen and liver in mice (Paczosa *et al.*, 2014). In the lungs, YadA and Ail target Yop injection at neutrophils, which is needed for growth in the lungs (Paczosa *et al.*, 2014).

2.4 Detection

Pathogenic *Yersinia*, including *Y. pseudotuberculosis*, grow slowly and are poor competitors, which makes their conventional isolation challenging (Fredriksson-Ahomaa & Korkeala, 2003, Skurnik *et al.*, 2009). The isolation of *Y. pseudotuberculosis* is facilitated by using a selective culture medium. *Yersinia* colony on cefsulodin-irgasan-novobiocin (CIN) selective agar has a sharply bordered deep red centre and a translucent outer zone (Schiemann, 1979). On MacConkey agar supplemented with sorbitol (SMAC), *Y. pseudotuberculosis* forms colourless colonies (Shiozawa *et al.*, 1991). Growth of *Y. pseudotuberculosis* on CIN agar (Schiemann, 1979) is slower than growth of other *Yersinia* species on the same agar, and thus, direct plating is not effective for the isolation of *Y. pseudotuberculosis* from samples with abundant background flora (Niskanen *et al.*, 2008, Niskanen *et al.*, 2002). Also overnight enrichment using trypticase soy broth followed by incubation in modified Rappaport broth (Wauters, 1973) or selective enrichment with Rappaport broth (Wauters, 1973) has been shown to be ineffective in isolating *Y. pseudotuberculosis* from biological samples (Niskanen *et al.*, 2002). Alkali treatment with potassium hydroxide can be used to increase the isolation level of *Y. pseudotuberculosis* from food samples (Aulisio *et al.*, 1980). Probably the most successful isolation method for *Y. pseudotuberculosis* from samples with abundant background flora is cold enrichment in phosphate-buffered saline supplemented with 1% mannitol and 0.15% bile salts followed by alkali treatment and plating on either CIN or SMAC selective agar plates (Martinez *et al.*, 2009, Niskanen *et al.*, 2008, Niskanen *et al.*, 2002, Ortiz Martínez *et al.*, 2010).

Y. pseudotuberculosis, *Y. similis*, and *Y. pekkanenii* have similar phenotypes in biochemical reactions and cannot be distinguished with biochemical tests (Murros-Kontiainen *et al.*, 2011a, Niskanen *et al.*, 2009, Sprague *et al.*, 2008). This is why PCR with primers targeted to *inv*, *virF*, and *yadA* is often used to detect and identify *Y. pseudotuberculosis* (Fredriksson-Ahomaa & Korkeala, 2003, Fredriksson-Ahomaa *et al.*, 2010, Skurnik *et al.*, 2009). Both single and multiplex PCR, either with gel detection or real-time detection are used (Fredriksson-Ahomaa & Korkeala, 2003, Fredriksson-Ahomaa *et al.*, 2010, Skurnik *et al.*, 2009).

2.5 Stress

Y. pseudotuberculosis is capable of growing at refrigeration temperatures and under modified atmospheres (Bottone *et al.*, 2005, Fredriksson-Ahomaa *et al.*, 2010). It grows at a pH range of 5.0 to 9.6 (Bottone *et al.*, 2005) and in 5% sodium chloride (Fredriksson-Ahomaa *et al.*, 2010). The ability to tolerate the common preservation methods of the modern food chain has increased the importance of this pathogen. *Y. pseudotuberculosis* can also survive for long period in soil (Fukushima *et al.*, 1989, Jalava *et al.*, 2006) and in food storage facilities (Rimhanen-Finne *et al.*, 2009).

A temperature decrease affects the metabolism and function of bacterial cells, with temperature being sensed by the cell membrane, nucleic acids, and ribosomes (Phadtare, 2004). Cell membranes are solidified, which influences cellular transport and secretion. Efficiency of transcription and translation is reduced due to stabilized secondary structures of RNA and DNA. In addition, low temperature increases negative supercoiling of DNA. This changes the positions of promoter regions, thus disturbing promoter recognition by the vegetative sigma factor (Phadtare, 2004). Furthermore, most mRNAs cannot be translated before cells are adapted to the low temperature (Jones & Inouye, 1996).

In addition to the physiological endogenous oxidative stress generated during aerobic growth due to chemical oxidation of electron carriers by molecular oxygen (Storz & Imlay, 1999), *Yersinia* can encounter oxidative stress in the environment, in foods stored at low temperatures, and in the host during the infection process. Numerous reactive oxygen species are formed endogenously in bacterial cells at low temperatures because electrons accumulate in the respiratory chain due to the slow metabolic rate (Dhar *et al.*, 2013, Na *et al.*, 2011). In addition, reactive oxygen species are produced by macrophages, and deletion of a superoxide dismutase encoding gene resulted in attenuated virulence of *Y. pseudotuberculosis* in mice (Champion *et al.*, 2009). Oxidative stress and reactive oxygen species damage DNA, cell membranes, and proteins and cause enzyme dysfunction in bacterial cells (Imlay & Linn, 1988, Storz & Imlay, 1999). *Y. pseudotuberculosis* is exposed to acid stress in foodstuffs and in the stomach, intestines, and macrophages of the host (Kanjee & Houry, 2013).

2.6 Stress tolerance mechanisms

Bacteria can use several mechanisms to adapt to a stressful environment. These include synthesis of protective enzymes and changes in the gene expression pattern. To safeguard against oxidative stress, bacteria even during normal growth produce several antioxidant enzymes to protect themselves from damage (Storz & Imlay, 1999). The superoxide dismutases SodA and SodB of *Y. enterocolitica* are reactive oxygen species-detoxifying enzymes that function optimally at 4°C and under acidic pH, facilitating the survival of the bacterium in the acidic conditions of the phagolysosome and at low temperatures (Dhar *et al.*, 2013). Production of urease, which catalyses the hydrolysis of urea to carbon dioxide and ammonia, increases the survival of *Y. pseudotuberculosis* in acidic culture media (Hu *et al.*, 2009a, Riot *et al.*, 1997). In contrast to *Y. enterocolitica*, in which production of

urease enhances survival of the pathogen in the host (De Koning-Ward & Robins-Browne, 1995), in *Y. pseudotuberculosis*, production of urease does not affect survival in the host, virulence, or LD_{50} in mice (Riot *et al.*, 1997).

When growth temperature changes, fatty acid composition of the bacterial cell membrane is altered (Suutari & Laakso, 1994). In *E. coli*, the thermosensor that senses temperature change and affects lipid composition of the cell membrane is situated in the cytosol (Mansilla *et al.*, 2004). At low temperatures, the amount of low-melting-point unsaturated fatty acids increases due to the activity of the FabF enzyme (Mansilla *et al.*, 2004). Accordingly, at higher growth temperatures there are more saturated fatty acids in the cell membrane (Suutari & Laakso, 1994). Similar to other bacteria, *Y. enterocolitica* and *Y. pseudotuberculosis* have more unsaturated fatty acids in their cell membranes when grown at low temperatures (Bakholdina *et al.*, 2004, Goverde & Kusters, 1994, Nagamachi *et al.*, 1991).

In *Y. enterocolitica*, different genes are expressed during the cold-shock response and during long-term adaptation to low temperature (Bresolin *et al.*, 2006). When growth temperature of *Y. enterocolitica* is decreased from 30°C to 10°C, genes encoding cold-shock proteins and a gene encoding glutamate-aspartate symporter are first induced (Bresolin *et al.*, 2006). After this cold-shock phase, cells continue to divide. At the early and middle exponential growth phases, genes encoding environmental sensors and regulators and genes related to motility and virulence, including insecticidal toxin genes and chemotaxis genes, are activated (Bresolin *et al.*, 2006). In the late exponential and early stationary growth phases, genes encoding biodegradative enzymes are induced (Bresolin *et al.*, 2006). Induction of biodegradative operons at low temperatures in psychrotrophic bacteria probably compensates for low enzyme activities at low temperatures, providing a sufficient level of nutrients (Kannan *et al.*, 1998).

2.6.1 Two-component signal transduction systems

Bacteria must sense, respond, and adapt to their environment to stay alive. For this, they can use two-component signal transduction systems (TCSs) (Capra & Laub, 2012). A classical TCS consists of a histidine kinase located in the cell membrane and a response regulator located in the cytoplasm (West & Stock, 2001). The histidine kinase senses the intra- or extracellular environment, e.g. the availability of nutrients, cellular redox state, osmolarity, and antibiotics (Laub & Goulian, 2007). When the histidine kinase senses a stimulus, it autophosphorylates. Next, the phosphoryl group is passed to the response regulator and the response regulator binds to DNA and affects transcription. Often histidine kinases can both phosphorylate and desphosphorylate their response regulator-pairs. Thus, following a stimulus, either phosphotransfer or phosphatase activity of the histidine kinase is induced, activating or inactivating the response regulator, respectively (Laub & Goulian, 2007). Most response regulators are transcription factors that can either induce or repress transcription of specific genes (Stock *et al.*, 2000). However, some response regulators function as enzymes and others use assistant proteins to influence, for example, cell motility (Stock *et al.*, 2000). A phosphorelay is a modification of a TCS

composed of a hybrid histidine kinase, a histidine phosphotransferase (Hpt), and a response regulator (Laub & Goulian, 2007). Hpt passes the phosphoryl group from the hydrid histidine kinase to the response regulator (West & Stock, 2001). Thus, many TCSs contain more than two components.

NtrB/NtrC TCS of *E. coli* was the first TCS described (Ninfa & Magasanik, 1986), and many homologous proteins were soon reported in *E. coli* and other bacteria (Nixon *et al.*, 1986). Thus, most bacteria have TCSs (Capra & Laub, 2012). On average, in eubacteria TCS proteins form 1% of encoded proteins (West & Stock, 2001). However, there is variation in the number of TCSs in different bacteria (Stock *et al.*, 2000). Whereas *Synechocystis sp.* encodes 80 TCS proteins, *Mycoplasma genitalium* encodes none (Stock *et al.*, 2000). The genome of *Y. pseudotuberculosis* IP32953 was found to contain 24 complete TCSs and 5 orphan hybrid histidine kinases or response regulators in in silico analysis (Marceau, 2005). Not all TCSs of *Y. pseudotuberculosis* have been investigated. However, several TCSs of *Y. pseudotuberculosis* play a role in virulence and/or in resistance to environmental stressors, including stresses encountered in the host (Table 2).

Table 2. Two-component signal transduction systems of *Yersinia pseudotuberculosis* that have been investigated.

Name	Component[1]	Function
NtrB/NtrC	HK/RR	Bile salt resistance (Flamez *et al.*, 2008)
CpxA/CpxR	HK/RR	Eukaryotic cell contact (Carlsson *et al.*, 2007b), type III secretion (Carlsson *et al.*, 2007a, Liu *et al.*, 2012), regulation of virulence gene expression during extracytoplasmic stress (Liu *et al.*, 2011)
PmrB/PmrA	HK/RR	Acid and bile salt resistance (Flamez *et al.*, 2008); role in peptidoglycan homeostasis possible (Marceau *et al.*, 2004)
ArcB/ArcA	HK, Hpt/RR	Hydrogen peroxide and bile salt resistance (Flamez *et al.*, 2008)
BarA/UvrY	HK, RR, Hpt/RR	Involved in the expression of virulence genes (Heroven *et al.*, 2008, Heroven *et al.*, 2012b)
RcsC/YojN/RcsB	HK, RR/Hpt/RR	Regulates cell envelope, virulence, motility, and biofilm formation (Hinchliffe *et al.*, 2008). Bile salt resistance in some strains (Hinchliffe *et al.*, 2008), bile salt susceptibility in others (Flamez *et al.*, 2008, Hinchliffe *et al.*, 2008)
RstB/RstA	HK/RR	Hydrogen peroxide resistance, virulence (Flamez *et al.*, 2008)

Name	Component[1]	Function
PhoQ/PhoP	HK/RR	Antimicrobial (Bozue *et al.*, 2011, Flamez *et al.*, 2008, Marceau *et al.*, 2004), acid, and osmotic stress resistance (Flamez *et al.*, 2008); virulence (Bozue *et al.*, 2011, Fisher *et al.*, 2007, Flamez *et al.*, 2008, Grabenstein *et al.*, 2004); survival and replication in macrophages (Bozue *et al.*, 2011, Grabenstein *et al.*, 2004); biofilm regulation (Sun *et al.*, 2009); hydrogen peroxide and bile salt susceptibility (Flamez *et al.*, 2008)
YfhK/YfhA	HK/RR	Virulence and susceptibility to antimicrobial agents (Flamez *et al.*, 2008), role in glucosamine-6-phosphate synthesis (Göpel *et al.*, 2011)
Envz/OmpR	HK/RR	Osmotic stress, acid and bile salt resistance (Flamez *et al.*, 2008, Hu *et al.*, 2009a); involved in flagella biosynthesis (Hu *et al.*, 2009b); antimicrobial agent susceptibility and virulence (Flamez *et al.*, 2008); regulation of type VI secretion system (Gueguen *et al.*, 2013, Zhang *et al.*, 2013)
CvgSY	HK, RR	Virulence (Karlyshev *et al.*, 2001)

[1]HK, histidine kinase; RR, response regulator; Hpt, histidine phosphotransferase; separate proteins of the system are segregated with /

A role of TCSs in cold stress has been demonstrated in bacteria. In *Clostridium botulinum*, TCSs *cbo*0365/*cbo*0366, *cbo*2306/*cbo*2307, and *clo*3403/*clo*3404 are essential for optimal growth at low temperatures (Derman *et al.*, 2013, Lindström *et al.*, 2012, Mascher *et al.*, 2014). In *Listeria monocytogenes*, TCSs LisK/LisR, *lmo*1061/*lmo*1060, and *lmo*1173/*lmo*1172 enhance growth immediately following a temperature drop, and are indispensable after cells have adapted to the low temperature (Chan *et al.*, 2008). In *Bacillus subtilis*, TCS DesK/DesR is involved in desaturation of membrane lipids in response to a temperature drop (Aguilar *et al.*, 1998, Aguilar *et al.*, 2001).

Despite the large number of TCSs in many bacteria, cross-talk between non-cognate histidine kinases and response regulators is rare (Groban *et al.*, 2009, Podgornaia & Laub, 2013). Cross-regulation, advantageous cross-talk, is seldom used in situations where combining multiple signals into one response or expanding one signal to many responses is beneficial (Laub & Goulian, 2007).

2.6.2 Sigma factors

In bacteria, a housekeeping sigma factor is used for transcription of most promoters (Österberg *et al.*, 2011). Alternative sigma factors are used for transcription of genes needed for adaptation to changing environmental conditions. The more variable the environment in which a bacterium lives, the more alternative sigma factors its genome encodes (Österberg *et al.*, 2011). Typically, alternative sigma factors are regulated stringently to hinder their competition with the housekeeping sigma factor under non-stress conditions (Battesti *et al.*, 2011). The genome of *E. coli* contains six alternative sigma factors in addition to the vegetative sigma factor, while the Gram-positive *Streptomyces coelicolor* has 63 sigma factors (Gruber & Gross, 2003). The genome of *Y. pseudotuberculosis* IP32953 encodes seven sigma factors (Chain *et al.*, 2004). The sigma factors of *E. coli* and *Y. pseudotuberculosis* strain IP32953 are listed in Table 3. The requirement of alternative sigma factors in stress conditions has been demonstrated in food pathogens *C. botulinum* (Dahlsten *et al.*, 2013) and *L. monocytogenes* (Mattila *et al.*, 2012, Raimann *et al.*, 2009). Alternative sigma factors σ^E, σ^S, FliA, and σ^{54} have been investigated in enteropathogenic *Yersinia*.

Table 3. Sigma factors of *Escherichia coli* and *Yersinia pseudotuberculosis*.

Sigma factor	Function in *E. coli*[1]	Locus tag in *Y. pseudotuberculosis* IP32953[2]
σ^{70} Family		
$\sigma^{70/D}$	Housekeeping sigma factor, essential	YPTB3418
$\sigma^{38/S}$	Stationary phase and general stress response sigma factor	YPTB0776
$\sigma^{28/FliA}$	Flagellar sigma factor	YPTB1715, YPTB3320
$\sigma^{19/FecI}$	Iron citrate uptake sigma factor	Not identified
$\sigma^{24/E}$	Extracytoplasmic stress sigma factor, essential	YPTB2897
$\sigma^{32/H}$	Heat-shock sigma factor	YPTB0224
σ^{54} Family		
$\sigma^{54/N}$	Nitrogen metabolism sigma factor, also involved in several different physiological processes	YPTB3526

[1]Reviewed by Buck *et al.* (2000), Gruber & Gross (2003), Hengge (2009), Wösten (1998), Österberg *et al.* (2011)
[2]Chain *et al.* (2004)

σ^E

An alternative sigma factor, σ^E is activated in *E. coli* when composition or folding of cell envelope proteins is disturbed due to heat (Erickson & Gross, 1989), increased expression of outer membrane proteins (Mecsas *et al.*, 1993), ethanol (Raina *et al.*, 1995), or high osmolality (Bianchi & Baneyx, 1999). In addition, transcription of *rpoE* encoding σ^E is induced during cold-shock response and during growth at low temperatures in *E. coli* (Moen *et al.*, 2009, Polissi *et al.*, 2003). σ^E is also involved in raising genetic diversity and allowing cell survival in a stringent environment via stress-induced mutagenesis in *E. coli* (Gibson *et al.*, 2010). In *E. coli* and in other *Enterobacteriaceae*, σ^E regulates transcription of genes associated with pathogenesis (Rhodius *et al.*, 2006). In addition, σ^E-dependent envelope stress response assists survival of many Gram-negative pathogens in the host, and σ^E is required for full virulence (Raivio, 2005).

In *E. coli*, *rpoE* is an essential gene (Baba *et al.*, 2006, De Las Penas *et al.*, 1997). At the amino acid level, *rpoE* encoding σ^E differs between *Y. pseudotuberculosis* IP32953 and *E. coli* K-12 by 7% (Blattner *et al.*, 1997, Chain *et al.*, 2004). In *Y. enterocolitica* and *Y. pseudotuberculosis*, σ^E has been proposed to be involved in pathogenesis and virulence (Carlsson *et al.*, 2007a, Heusipp *et al.*, 2003, Young & Miller, 1997).

σ^S

In *E. coli* and other γ-proteobacteria, σ^S regulates general stress response (Hengge, 2009). σ^S controls up to 10% of all of the genes of *E. coli* (Weber *et al.*, 2005). σ^S is induced in *E. coli* in several stress conditions, including beginning of the stationary growth phase, hyperosmolarity, high or low temperature, acidic or alkaline pH, and high cell density (Allen *et al.*, 2008, Battesti *et al.*, 2011, Moen *et al.*, 2009, Sledjeski *et al.*, 1996, White-Ziegler *et al.*, 2008). A hairpin loop in the 5'untranslated region of *rpoS* mRNA inhibits its translation when σ^S is not needed. Small RNAs (sRNA) together with an RNA chaperone protein Hfq open the hairpin and the *rpoS* mRNA can be translated (Battesti *et al.*, 2011). At a low temperature (24°C), an additional factor, DEAD-box helicase CsdA, is needed for σ^S synthesis in *E. coli* (Resch *et al.*, 2010). CsdA opens the hairpin loop structure of the *rpoS* mRNA, allowing sRNA DsrA (Sledjeski *et al.*, 1996) to bind to the *rpoS* mRNA and induce its translation at low temperatures in *E. coli* (Resch *et al.*, 2010). A σ^S-mediated stress response activated because of one stress condition gives the bacterium cross-protection against other stress conditions as well (Battesti *et al.*, 2011). The purpose of this cross-protection is to prevent cell damage (Hengge-Aronis, 2002). Also stress resistance mechanisms overlap. Transcription of *otsA* and *otsB* encoding trehalose-synthesizing enzymes requires σ^S at a low temperature (16°C) in *E. coli* (Kandror *et al.*, 2002). During the cold-shock response *E. coli* cells synthesize and accumulate disaccharide trehalose, which increases cell viability at temperatures near 0°C (Kandror *et al.*, 2002). The σ^S-dependent expression of *otsA* and *otsB*, and thus, accumulation of intracellular trehalose, is also needed for resistance to high osmolarity

and high temperature (Battesti *et al.*, 2011). In addition, σ^S controls the expression of several virulence genes in pathogenic *Enterobacteriaceae* (Hengge-Aronis, 2002).

In *Y. enterocolitica* grown at 37°C, mutation of *rpoS* encoding σ^S reduces survival from stress caused by hydrogen peroxide, high osmolarity, high temperature, and low pH (Badger & Miller, 1995). Mutation affects also cell morphology at 37°C. At 26°C mutation does not have effects on stress survival or cell morphology (Badger & Miller, 1995). In addition, mutation of *rpoS* does not affect Inv or Ail levels (Badger & Miller, 1995), invasion *in vitro* (Badger & Miller, 1995) or *in vivo* (Iriarte *et al.*, 1995b), or LD_{50} (Badger & Miller, 1995, Iriarte *et al.*, 1995b). The function of σ^S of *Y. pseudotuberculosis* has not been investigated. The gene *rpoS* encoding σ^S is 99% similar at the amino acid level in *Y. pseudotuberculosis* IP32953 and *Y. enterocolitica* 8081 (Chain *et al.*, 2004, Thomson *et al.*, 2006).

FliA

σ^{28} or FliA is a flagellar sigma factor that positively controls expression of class III flagellar genes encoding components of flagellar filament and hook-associated, motor, and chemotaxis proteins (Ding *et al.*, 2009, Soutourina & Bertin, 2003). In *Y. enterocolitica*, *fliA* encoding FliA is essential for motility (Iriarte *et al.*, 1995a). In *Y. enterocolitica*, the expression of flagellar genes is maximal at 20°C (Bresolin *et al.*, 2008), and flagellin genes (Kapatral & Minnich, 1995) and *fliA* (Kapatral *et al.*, 1996) are not transcribed at 37°C. In *Y. pseudotuberculosis*, at 22°C *fliA* is expressed 25-fold more than its expression at 37°C (Atkinson *et al.*, 2008). Thus, *Y. enterocolitica* and *Y. pseudotuberculosis* are non-motile at temperatures higher than 30°C (Marceau, 2005). Deletion of *fliA* does not affect pathogenicity of *Y. enterocolitica* (Iriarte *et al.*, 1995a), even though FliA controls positively transcription of *yplA* encoding a virulence factor phospholipase YplA (Schmiel *et al.*, 2000) and negatively several virulence genes encoded in pYV through VirF at 25°C (Horne & Prüß, 2006) in *Y. enterocolitica*. In *Y. pseudotuberculosis*, expression of *yplA* is not controlled by the flagellar regulon (Meysick *et al.*, 2009).

σ^{54}

The σ^{54} family of sigma factors has only one member, sigma factor σ^{54} (Wösten, 1998). The sigma factors of this family have no homology with the sigma factors of the σ^{70} family (Reitzer, 2003). In addition to the genes associated with nitrogen metabolism, σ^{54}-regulated genes include genes involved in tolerance of acid and alkaline stress and universal stress response in E. coli (Reitzer, 2003). In *Y. pseudotuberculosis*, σ^{54} and response regulator YfhA of TCS YfhK/YfhA control transcription of sRNAs GlmY and GlmZ involved in the production of glucosamine-6-phosphate, and thus, cell wall and outer membrane synthesis (Göpel *et al.*, 2011).

2.6.3 Cold-shock proteins and cold-induced proteins

When temperature suddenly decreases by at least 10°C, the cold-shock response is induced (Jones *et al.*, 1987). During this response, while general protein synthesis decreases and bacteria stop dividing, small cold-shock proteins (Csps) and other cold-induced proteins (Cips) are synthesized (Phadtare, 2004). *E. coli* encodes nine Csps of which CspA, CspB, CspE, CspG, and CspI are induced at low temperatures (Phadtare, 2004, Uppal *et al.*, 2008). In *E. coli*, Csps can replace each other (Phadtare, 2004, Xia *et al.*, 2001). At low temperatures, Csps destabilize the secondary structures of RNA and they are RNA chaperones, which helps transcription and translation, and thus, growth (Brandi *et al.*, 1999, Jiang *et al.*, 1997, Phadtare, 2004). Cips of *E. coli* include proteins involved in transcription (Gualerzi *et al.*, 2003, Jones *et al.*, 1987, La Teana *et al.*, 1991, Madrid *et al.*, 2001), translation (Agafonov *et al.*, 1999, Agafonov & Spirin, 2004, Giangrossi *et al.*, 2007, Giuliodori *et al.*, 2004, Gualerzi *et al.*, 2003, Jones *et al.*, 1996, Jones *et al.*, 1987, Xia *et al.*, 2003), RNA metabolism (Jones *et al.*, 1996, Jones *et al.*, 1987), homologous recombination and SOS response (Gualerzi *et al.*, 2003, Jones *et al.*, 1987), cellular metabolism (Jones *et al.*, 1987), and chromosome replication (Atlung & Hansen, 1999). The purpose of Csp and Cip production is to adapt cells to the new temperature and to promote cell growth (Brandi *et al.*, 1999, Jiang *et al.*, 1997, Phadtare, 2004). After adaptation to the low temperature, Csp and Cip synthesis decreases and cells grow at a lower rate (Jones *et al.*, 1987).

In addition to Csp synthesis during cold shock, Csps can be produced throughout the growth cycle or during nutriotional upshift (Brandi *et al.*, 1999, Yamanaka & Inouye, 2001). Furthermore, CspC and CspE are involved in σ^S-mediated general stress response in *E. coli* (Battesti *et al.*, 2011). Overexpression of CspC and CspE stabilizes *rpoS* mRNA and increases the amount of σ^S (Phadtare & Inouye, 2001), while deletion of *cspC* decreases *rpoS* expression (Cohen-Or *et al.*, 2010). CspC and CspE are also required for proper expression of σ^S during osmotic stress (Phadtare & Inouye, 2001).

Csps are highly conserved proteins (Graumann & Marahiel, 1998). They contain a cold-shock domain (CSD), which is very similar to a domain present in nucleic-acid-binding proteins of eukaryotes (Graumann & Marahiel, 1998). This means that a common ancestor of eukaryotes and prokaryotes 3.5 billion years ago probably had a CSD protein (Graumann & Marahiel, 1998). Several Gram-positive and Gram-negative bacteria produce Csps, but Csps are lacking in archaea and cyanobacteria (Phadtare, 2004).

Cip CsdA is a cold-induced RNA helicase (Jones *et al.*, 1996) belonging to the highly conserved family of DEAD-box (asp-glu-ala-asp) proteins (Linder *et al.*, 1989). The need for CsdA in *E. coli* is higher at 15°C than at 25°C, and deletion of *csdA* impairs growth at temperatures below 25°C (Awano *et al.*, 2007, Charollais *et al.*, 2004, Jones *et al.*, 1996, Turner *et al.*, 2007). The importance of DEAD-box helicases during cold stress has also been demonstrated in *L. monocytogenes* and *Bacillus cereus* (Azizoglu & Kathariou, 2010, Markkula *et al.*, 2012, Netterling *et al.*, 2012, Pandiani *et al.*, 2010). Secondary structures of RNA are stabilized at low growth temperatures, but by unwinding double-stranded RNA, CsdA facilitates translation initiation at cold temperatures in *E. coli* (Jones *et al.*, 1996, Lu *et al.*, 1999). CsdA is involved in the degradation of mRNAs in *E. coli*

(Khemici *et al.*, 2004, Prud'homme-Généreux *et al.*, 2004, Yamanaka & Inouye, 2001). In addition, at low temperatures it is needed for the biogenesis of 50S ribosomal subunits (Charollais *et al.*, 2004, Peil *et al.*, 2008) and also at 37°C in the early exponential growth phase in *E. coli* (Peil *et al.*, 2008).

Regulation of Csp and Cip synthesis

The most significant regulation of protein synthesis during the cold-shock response takes place at the post-transcriptional and translational levels (Goldenberg *et al.*, 1997, Gualerzi *et al.*, 2003). Csp and Cip mRNAs are very stable immediately after a temperature downshift (Goldenberg *et al.*, 1996, Mitta *et al.*, 1997, Uppal *et al.*, 2008, Xia *et al.*, 2002). In addition, at low temperatures, translation of most mRNAs is reduced, but Csp and Cip mRNAs are translated efficiently (Jones & Inouye, 1996). This translational bias is due to the structure of the mRNAs; the long 5' untranslated region (5'UTR) of CspA mRNAs has an affinity to ribosomes (Xia *et al.*, 2002), and secondary and tertiary structures of the 5'UTRs of Csp and Cip mRNAs facilitate translation (Giuliodori *et al.*, 2004, Uppal *et al.*, 2008, Yamanaka *et al.*, 1999). Furthermore, increased synthesis of initiation factors after a temperature downshift favours the translation of Csp and Cip mRNAs (Giangrossi *et al.*, 2007, Giuliodori *et al.*, 2004, Giuliodori *et al.*, 2007). Thus, translation of mRNAs other than those of Csp or Cip is hindered during the cold-shock response.

Cold-shock proteins of Yersinia

The genomes of enteropathogenic *Yersinia* have several *csp* genes (Palonen *et al.*, 2010). Two of them, *cspA1* and *cspA2*, are almost identical and are located next to each other (Neuhaus *et al.*, 1999). In *Y. enterocolitica*, both monocistronic and bicistronic mRNAs are produced from these loci, enabling rapid adaptation of this bacterium to low temperatures (Neuhaus *et al.*, 1999). Because Csp and Cip mRNAs hold ribosomes, and thus, block the translation of other mRNAs, in order to resume growth of the cell population, Csp and Cip mRNAs must be degraded (Neuhaus *et al.*, 2000). As demonstrated in *Y. enterocolitica*, cell division restarts when the amount of *cspA1/A2* transcripts has decreased sufficiently (Neuhaus *et al.*, 2000). To enable efficient degradation of Csp mRNAs after the cold-shock response, *cspA1/A2* mRNAs of *Y. enterocolitica* have special cleavage sites for RNase E, which begins the degradation of the transcripts. After cleavage by ribonuclease (RNase) E, polynucleotide phosphorylase (PNPase) continues *cspA1/A2* transcript degradation (Neuhaus *et al.*, 2003). For *Y. enterocolitica* and *Y. pseudotuberculosis*, PNPase is essential for growth at low temperatures (Goverde *et al.*, 1998, Rosenzweig *et al.*, 2005).

3 AIMS OF THE STUDY

The general objectives of this study were to investigate the genetic variability of virulence genes used in the detection and identification of *Y. pseudotuberculosis* and to evaluate the stress tolerance mechanisms of the pathogen.

Specific objectives were as follows:

1. To investigate the genetic variability of virulence genes *inv*, *virF* and *yadA* of different *Y. pseudotuberculosis* and *Y. similis* strains (Study I).

2. To assess the role of TCSs of *Y. pseudotuberculosis* IP32953 in cold tolerance (Study II).

3. To investigate the role of σ^E of *Y. pseudotuberculosis* IP32953 in cold, heat, osmotic, ethanol, acid, and alkaline stress (Study III).

4. To evaluate the role of RNA helicase CsdA in cold tolerance in *Y. pseudotuberculosis* IP32953 (Study IV).

4 MATERIALS AND METHODS

4.1 Bacterial strains, plasmids, and growth conditions (I-IV)

The *Y. pseudotuberculosis* and *Y. similis* strains used in Study I are listed in Table 4. All of the genetic modifications were done in strain IP32953, a generous gift from Elisabeth Carniel, Institut Pasteur, Paris, France. The strains and plasmids used in Studies II-IV are presented in Table 5.

Bacteria were grown on Luria-Bertani (LB) agar plates (BD, Franklin Lakes, NJ, USA) or in LB broth (BD). *Y. pseudotuberculosis* and *Y. similis* were cultivated at 30°C, at 28°C, or at 37°C, and *E. coli* at 37°C. *Y. pseudotuberculosis* was grown at 3°C and at 42°C in cold and heat stress experiments, respectively. In other stress experiments, the pH of the broth was adjusted to 5.0 (with 1M HCl) or 9.0 (with 1M NaOH), or contained 30 g/l NaCl or 3% ethanol. Culture media were supplemented with 5 mM $CaCl_2$ to inhibit the release of Yops at 37°C and 42°C (Mulder *et al.*, 1989), or with antibiotics (Sigma-Aldrich Co., St. Louis, MO, USA) when appropriate. Plate count agar (PCA) (Sigma-Aldrich Co.) was used in the verification of the correspondence of optical density at 600 nm (OD_{600}) values and the number of viable bacteria. Tryptic soy agar (TSA) (BD) containing 1.5% or 2.5% agar was used to determine the minimum and maximum growth temperatures, respectively.

4.2 PCR amplification, sequencing, and sequence analysis (I)

The sequenced strain IP32953 (Chain *et al.*, 2004) was used in primer design using Primer3 software (Rozen & Skaletsky, 2000) (I, Table S1). Pitcher's method (Pitcher *et al.*, 1989) was used to extract the genomic DNA from the *Y. pseudotuberculosis* and *Y. similis* strains, and the Qiagen Plasmid Midi Kit (Qiagen GmbH, Hilden, Germany) was used for extraction of plasmid DNA according to the manufacturer's instructions. PCR was performed with the Titanium Taq DNA polymerase (Clontech Laboratories Inc., Mountain View, CA, USA) following the manufacturer's instructions. Each reaction contained 200 µM of dNTPs, 0.3 µM of both primers, 100 ng of DNA template, and had a total volume of 50 µl. The cycling protocol for products under 1000 bp included predenaturation at 95°C for 1 min, and 29 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min, and a final extension at 72°C for 2 min. Extension times were 90 s and 2 min for products between 1000 and 1500 bp and between 1500 and 2000 bp, respectively. The PCR products were visualized in agarose gel, and the desired products were purified by using the QIAquick Gel Extraction Kit (Qiagen GmbH) according to the manufacturer's instructions. Sequencing was performed at the Institute of Biotechnology, University of Helsinki, Finland.

The Phred (Ewing & Green, 1998) was used in base-calling and quality assignment of the raw sequences, and the gap4 program in the Staden package (Staden, 1996) was used

to construct gene sequences. Phylogenetic trees were created using MEGA (Tamura *et al.*, 2007), and alignment of sequences was done with CLUSTAL. The Kimura 2-parameter model for nucleotide sequences was used to build neighbour-joining trees with 1000 bootstrap replicates. No evolution model was used in calculation of pairwise sequence similarities.

Table 4. *Yersinia pseudotuberculosis* and *Yersinia similis* strains used in Study I.

Strain	Origin	Country	Source[1]
Y. pseudotuberculosis			
H346-36/89	Human	Germany	M. Skurnik
WS899K	Capybara	Germany	DFHEH
YLI16.9	Song thrush	Sweden	DFHEH
LE116.1K	Cat	Finland	DFHEH
A162.1K3	Lettuce	Finland	DFHEH
553	Soil	Russia	M. Skurnik
P30	Buffalo	Brazil	M. Skurnik
S107	Rabbit	China	M. Skurnik
476	Pig	Italy	M. Skurnik
43K.3	Pig	England	DFHEH
H5N6.1.1K2D	Pig	Belgium	DFHEH
T3.2.2K	Pig	England	DFHEH
T1.4.1K	Pig	England	DFHEH
14994/83	Human	Finland	M. Skurnik
T6.2.2C	Pig	England	DFHEH
2B1	Roasted fish	Nigeria	DFHEH
$S_5$3B1-3/IIIC	Shrew	Finland	DFHEH
283	Human	Canada	M. Skurnik
Y. similis			
R626R	Mole	Japan	M. Skurnik
R220	Field mouse	Japan	M. Skurnik

[1]Mikael Skurnik, Haartman Institute, Department of Bacteriology and Immunology, University of Helsinki, and HUSLAB, Helsinki University Central Hospital, Finland; DFHEH, Department of Food Hygiene and Environmental Health, University of Helsinki, Finland

Table 5. Bacterial strains and plasmids used in Studies II-IV.

Strain	Source[I]	Characteristic
IP32953	E. Carniel	Parental strain in genetic modifications
*cheA*30	II	Insertional mutant IP32953 *cheA*30-31::Ltr Kan^r in sense orientation
*cheA*243	II	Insertional mutant IP32953 *cheA*243-244::Ltr Kan^r in antisense orientation
*cheY*243	II	Insertional mutant IP32953 *cheY*243-244::Ltr Kan^r in sense orientation
*rpoE*475	III	Insertional mutant IP32953 *rpoE*475-476::Ltr Kan^r in antisense orientation
*csdA*483	IV	Insertional mutant IP32953 *csdA*483-484::Ltr Kan^r in antisense orientation
*csdA*1548	IV	Insertional mutant IP32953 *csdA*1548-1549::Ltr Kan^r in sense orientation
*csdA*1578	IV	Insertional mutant IP32953 *csdA*1578-1579::Ltr Kan^r in antisense orientation
IP32953/pAR1219	II-IV	Parental strain with a source of T7 RNA polymerase
*rpoE*475/pBlue-*tetR-rpoE*	III	Mutant with the complementation plasmid
*rpoE*475/pBlue-*tetR*	III	Mutant with the empty complementation vector
IP32953/pBluescript II KS+	IV	Parental strain with the empty complementation vector
IP32953/pBluescript-*csdA*	IV	Parental strain with the complementation plasmid
*csdA*483/pBluescript-*csdA*	IV	Mutant with the complementation plasmid
*csdA*483/pBluescript	IV	Mutant with the empty complementation vector
*csdA*1548/pBluescript-*csdA*	IV	Mutant with the complementation plasmid
*csdA*1548/pBluescript	IV	Mutant with the empty complementation vector
*csdA*1578/pBluescript-*csdA*	IV	Mutant with the complementation plasmid
*csdA*1578/pBluescript	IV	Mutant with the empty complementation vector
Escherichia coli DH5α	Sigma-Aldrich Co.	Chemically competent cloning strain
Plasmids		
pAR1219	Sigma-Aldrich Co.	Source of T7 RNA polymerase
pACD4K-C	Sigma-Aldrich Co.	TargeTron vector
pACD4K-C-*cheA*30	II	TargeTron vector with intron targeted to *cheA* between bases 30 and 31 in sense orientation
pACD4K-C-*cheA*243	II	TargeTron vector with intron targeted to *cheA* between bases 243 and 244 in antisense orientation

Strain	Source[1]	Characteristic
pACD4K-C-*cheY*243-244	II	TargeTron vector with intron targeted to *cheY* between bases 243 and 244 in sense orientation
pACD4K-C-*rpoE*475	III	TargeTron vector with intron targeted to *rpoE* between bases 475 and 476 in antisense orientation
pACD4K-C-*csdA*483	IV	TargeTron vector with intron targeted to *csdA* between bases 483 and 484 in antisense orientation
pACD4K-C-*csdA*1548	IV	TargeTron vector with intron targeted to *csdA* between bases 1548 and 1549 in sense orientation
pACD4K-C-*csdA*1578	IV	TargeTron vector with intron targeted to *csdA* between bases 1578 and 1579 in antisense orientation
pBluescript II KS+	Stratagene	Cloning vector, complementation vector
pBlue-*tetR*	III	Cloning vector, complementation vector
pBlue-*tetR-rpoE*	III	Complementation plasmid containing *rpoE, rseA* and *rseB*, and 500 bp upstream of the start codon of *rpoE*
pBluescript-*csdA*	IV	Complementation plasmid containing *csdA* and 500 bp upstream of the start codon of *csdA*

[1]Elisabeth Carniel, Institut Pasteur, Paris, France

Table S1. Primers used in Study I. Start site is reported in relation to the start codon of strain IP32953. Table derived from Study I.

Gene (locus tag)	Primer	Direction [1]	Sequence	Start site
inv (YPTB1668)	1	F	TTGTGGCCAGTTATACCTCAAA	-100
	2	R	TTAAGTGTGTTGCGGCTGTC	596
	3	F	TGGCTCCTTGGTATGACTCTG	515
	4	R	GTTGCTGGCGATAATGTCAGT	1208
	5	F	TTTAATCTGCCCAGTCTGGTTT	-229
	6	R	GGTGACGCTCAATGTGAATG	1488
	7	R	AACGGTGATTGCAGTTTTCC	1569
	8	F	GGAGTGATGCCGAACTGATT	1286
	9	R	AGCTATCTGGCTGCTCGGTA	2275
	10	F	AGTTTCACCGTCTCCACACC	2104
	11	R	CAACGCCGTTCAATTAACCT	3098
	12	F	GCTCGTTTGTGGCCAGTTAT	-107
	13	R	CTGGGCGCTCTTTATAGTCG	802
	14	F	TAAAGACAGCCGCAACACAC	573
	15	R	AAAGTTGGCTGTCAGGTCGT	1419
	16	F	TTAAGATTAACGCCGCATCC	-974
	17	R	CTGGGCGCTCTTTATAGTCG	802
	18	F	AGTTTCACCGTCTCCACACC	2104
	19	R	CAAGCATTGGCAGTCTTGAA	3239
	20	F	GGTTGGCTGTACGGACTTAATAC	628
	21	R	AGCTATCTGGCTGCTCGGTA	2275
virF (pYV0076)	22	F	TTGGTTGCATTAATCGATGGT	-104
	23	R	TTTGATGGAGGTCGTTTCTTG	1025
	24	F	GGTTGTACATCGCACGCATA	-86
	KvirFL[2]	F	TCGTGGCAGCTATGCTGTTC	162
	KvirFR[2]	R	ATACGTCGCTCGCTTATCCA	653
	25	F	TCTCTTTTCCAGAGCGAGGA	-248
	26	R	TTTCGTTGAAATTTGGCTCA	915
	27	F	GGTTGTACATCGCACGCATA	-86
	28	R	AAATTTGGCTCATCCCATTG	907
yadA (pYV0013)	29	F	TTCTATGGGAGGCGTTCG	-97
	30	R	AGCGACACCAGTATCCGAAG	525
	31	F	TTGGGAGATTCGGCAGTTAC	427
	32	R	GCTGTTTAAAGCGGCTGAAC	1113
	33	F	TCGCGCAATTAAAGAAAGAAA	731
	34	R	CCCATGTAACTGAAACCATGATAA	1426
	35	F	CCTCGTTTGTCAGCAGTTCA	106
	36	R	TGAGCCAAAGTCTCTTTACGTG	794
	37	F	CTCTTCTATGGGAGGCGTTC	-100
	38	R	CCCGTAGCAAATATCGGAGA	1487
	39	F	TATCCGGTTTGAGGTGAGGA	-311
	40	F	GTGGATAACGCTCGACCACT	-627

Gene (locus tag)	Primer	Direction [1]	Sequence	Start site
	41	R	TTCAGCAGTAGCACCAATCG	339
	42	F	CTTAAAGCCGGTGTGGCTTA	1228
	43	R	AATCGCTTCACGTTCTGGAT	1943
	44	F	CCTCGTTTGTCAGCAGTTCA	106
	45	R	TCGGTGTTTTCCTATAGACTTGT T	1338
	46	F	CGGTGTACCCGTTTATGGTT	-700
	47	R	ACATAAATCGGATGCCCGTA	1501
	48	F	CTTCTATGGGAGGCGTTCG	-98
	49	R	CGCTGCTTCAGCAGTAGAAC	345

[1] F, forward; R, reverse
[2]Kaneko *et al.* (1995)

4.3 RNA isolation (II-IV)

To investigate the expression of genes encoding TCSs and *csdA*, *Y. pseudotuberculosis* IP32953 was grown to the early logarithmic growth phase at 3°C and 28°C. Expression of *rpoE* was investigated at early and late logarithmic growth phases at 3°C, 28°C, 37°C, and 42°C, and under acid, alkaline, osmotic, and ethanol stress. Bacteria were grown as three biological replicates in all experiments. Cells were collected by mixing bacterial culture with a cold phenol-ethanol mixture (1:9), kept on ice for 30 min, and centrifuged at 2°C at 5000×g for 15 min. Before RNA isolation, cell pellets were stored at -70°C. The total RNA was isolated using the RNeasy kit (Qiagen GmbH) with the RNase-free DNase set (Qiagen GmbH) following the manufacturer's instructions. The second DNase treatment was done using the DNA-free kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) and the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA) were used to determine the A260/A280 ratio and the integrity of RNA, respectively. The isolated RNA was kept at -70°C before use.

4.4 Reverse transcription (II-IV)

The RNA samples were reverse-transcribed into cDNA in duplicate by using the Dynamo cDNA synthesis kit (Thermo Scientific) following the manufacturer's instructions. Secondary structures of RNA were destabilized by incubating RNA for 5 min at 65°C. The reaction mixture containing 300 ng of random hexamers, 900 ng of RNA, and 2 µl of M-MuLV RNase H+ reverse transcriptase had a total volume of 20 µl and was incubated at 25°C for 10 min, at 37°C for 30 min, and at 85°C for 5 min. Minus-RT controls, having all the reaction components except the reverse transcriptase, were prepared from all RNA samples. The resulting cDNAs were stored at -20°C until use.

4.5 Quantitative real-time reverse transcription-PCR (II-IV)

In quantitative real-time reverse transcription-PCR (RT-qPCR), the Dynamo Flash SYBR Green qPCR kit (Thermo Scientific) was used according to the manufacturer's instructions. Primer3 software (Chain *et al.*, 2004, Rozen & Skaletsky, 2000) was utilized to design the primers (Table 7). Each reaction contained 4 µl of template cDNA, 0.5 µM of both primers, and had a total volume of 20 µl. The Rotor-Gene 3000 Real-Time Thermal Cycler (Qiagen GmbH) was used in PCR runs with the cycling protocol consisting of initial denaturation at 95°C for 10 s, annealing at 60°C for 15 s, extension at 72°C for 20 s, and a final extension at 60°C for 1 min. At the end of each extension step, fluorescence data were acquired. To confirm specificity, a melt curve analysis was done after each run. A dilution series of pooled cDNA was used in triplicate to determine the amplification reaction efficiency of each primer pair. The threshold fluorescence level for each primer pair and reaction efficiency as $10^{-\frac{1}{M}} - 1$, where M is the slope of the straight line from a semilogarithmic plot of the quantification cycle (C_q) as a function of the cDNA concentration, were defined with the Rotor-Gene 3000 software. Reaction efficiencies varied between 0.86 and 1.11. The cDNA dilutions 1:20 and 1:100000 were used in duplicate in the PCR amplification of the genes of interest and 16S *rrn*, respectively. The relative expression levels of investigated genes were normalized to 16S *rrn* and calibrated to samples taken at 28°C. Samples grown at 28°C with 5 mM $CaCl_2$ were used in calibration for samples collected at 37°C and 42°C. The gene expression levels were quantified by calculating the expression ratios (R) with the equation $R = \frac{\left(1 + E_{gene}\right)^{\Delta C_{q,gene}(\text{calibrator}-\text{sample})}}{\left(1 + E_{16Srrn}\right)^{\Delta C_{q,16Srrn}(\text{calibrator}-\text{sample})}}$ (Pfaffl, 2001), where E_{gene} is the amplification reaction efficiency of the transcript of the gene of interest, E_{16Srrn} is the amplification reaction efficiency of 16S *rrn* transcripts, $\Delta C_{q,gene}$ is the C_q deviation between calibrator and sample for the transcript of the gene of interest, and $\Delta C_{q,16Srrn}$ is the C_q deviation between calibrator and sample for the 16S *rrn* transcripts. To evaluate the significance of the differences between the relative expression levels of the gene of interest under different growth conditions, Student's t test was used.

Table 7. Primers used in quantitative real-time reverse transcription-PCR.

Gene	Forward primer (5'→3')	Reverse primer (5'→3')	Reference
16S rRNA gene	GCTCGTGTTGTGAAATGTTGG	TATGTGGTCCGCTGGCTCT	II-IV
ntrB	TCGTGTTGATGCGAGAGA	GTGCGGTCAGCGATAAAA	II
ntrC	CGTTTGGCTCGCTACTTTTT	CGCTTCGGTCTCTGGATG	II
cpxA	ATGGTCGGTCCCTTTTCC	ACATCATCAGCCGCATTTTT	II
cpxR	TGGAGTGAACAGCAACAAAA	AGCAGATAGAGCAGGGTGAA	II
envZ	CGTGAGATTTACCGTGAGTTGG	GCGACAGCCAGGTTTTGAG	II
ompR	ACGGAGCAAGGTTTTCAGGT	CCAACGATACGGTCCACTTCT	II
arcB	CACGGAAGAAGCACACAAAA	GCCCACTCACGCAATACC	II
arcA	TGAGCAGTGTAGGCGAAGAA	TGCGTGGCAGTTTGTAATG	II
creC	CGTTGGAATGATGTCTGGTTG	ATCTTGCGTTCACTGCGTTT	II
creB	AGCCCGTAGCGAAGAGTTG	AACCGCCAATAAGTGTGATTTT	II
phoQ	GCGACCTCTCGTTTACCTC	TGTTCACGCTCACCTTTTTC	II
phoP	GCCCTTCCAGATTGACCTTT	ACGACTTTCCCTGCGTTG	II
cheA	GGCGAAAGCACAGTCTCAA	ACATCCATCCCGACACCAC	II
cheY	AATGTGGAAGAAGCCGAAGA	TCACCATCAGAACAGGCAAC	II
barA	CGCAGTTATTCCAGGCTTTC	TTACCGCCCATTTCTTTCAC	II
uvrY	CAGCGTTCTTCTTGTTGATG	GCGACTTTGATACCTTTGATG	II
rstB	GGAATAAAACCATCGCAACA	CCAACCGCCAGAACATAG	II
rstA	ACCCTGTGCCGTGACCTAC	GCTGGCGGTGTCGTTTTT	II
kdpD	TTCGGTGTGATGCTGATTGT	GCCTGAAAACTGCTGGAAAG	II
kdpE	TGGCGAGTATTTGAGAGTGAG	CTTCATTGTTACGGGCAGA	II
baeS	GCCCACCTGCCTGAACAC	CAAATCGCCAACCCCAAC	II
baeR	AATCTACGGCGGAAACTG	ACAACAAACGGCACATCTCT	II
yfhK	GGCACAGCGTATCATTTGG	ATCGCCACCACCTCTTGT	II
yfhA	AAGCGATGGCAAAGAATGAA	GCCGACTCCCGTAATAGATG	II
phoR	CTGCCTGATGCCGTAGTT	TATGCTGCCCATTATCCTCT	II

Gene	Forward primer (5'→3')	Reverse primer (5'→3')	Reference
phoB	CCGTGGCGAAGAAGAAGA	CCTGTGAGATGAAGGGTCAAG	II
rcsC	ATGTGGCGATTTGTGTGTTG	TTTGATTGACTGGCTTGCTCT	II
yojN	CAGCAAGCAAGGCGTGAG	GCAAATCGGTAGTGTCCTGTAATG	II
rcsB	CCGCATTACAAAAAGGGAAG	TTCAGCAAAGAGTCGCAAAA	II
uhpB	CTGGGTGATGTCCTGTTGTG	CCATTCGCTCTCATTTTGCT	II
uhpA	CTTCAGGGATGGGGGTCA	GCCTTCAGCCAACAGCAC	II
pmrB	CCTTTCCCCCTTGGTTGTT	GGCGTATTCCTGCGAGTG	II
pmrA	ATCAGGGGCAGAGTGACAAC	AGACGTGAGAGGATCGCAAA	II
hydH	CGCAATAATACCCATCAGTCC	GCATCGCATCTAACGCTT	II
hydG	AAAACTCCTGCGTGCTATCC	CTTCGCTTCGCTCTCTCAAC	II
copS	GGACTCGGGCTCTCCATC	TCTGGCTACTGGCAAACACA	II
copR	GTATCCAGGCGTTGTTGAGG	ACAGGGCGAACTCTTTGG	II
evgS	GGAGAAGGTGTTCGCAGTTT	GATTGGGCTATTGAGGAGGAG	II
evgA	GCAGTGAAGCATTGGAAAAA	GCAACGAGAAACCATCAAAA	II
yehU	ACAGATTTTGGCGGGAGA	CATCGTGGCTGCGTTTTAG	II
yehT	TTTGGCGAAGACACTGACAC	CTGAACAGGGGATATGACGAA	II
narX	ACCAAACCATCAAGCCATTC	TGCCCTATCCCACAACCA	II
narP	CAAAGATTGCGAGCCAGAA	ACATCCCACGAGCCACCT	II
YPTB2728	CTACCAAAGCGGCAGTGAA	AGCCCACCCCAGAGATAAAG	II
YPTB2729	GCAAACGCCCATTATTTTTC	AGTCGTCAGCACCCAACTCT	II
YPTB2718	TGATAAACATCGCCGTGAAA	TAAATCCCCCTGTGCTACCC	II
YPTB2719	ATGCCTAAACCGTGCTATCC	TTACCCTGCCATTCGCTAC	II
YPTB0311	TCC CTTTCGCTCTGGTTATTT	CGCTCCTCCACTTTCATTTCT	II
YPTB0310	GAAGAAAAGTCCCCAGCAAA	AGAAAGTTGAGCAGCAATAACC	II
YPTB3808	ACCCCGCCTCTCCTTCTT	CACCGCTACGCCACTCAC	II
YPTB3801	CTGTGAAGTGCTGCGAGATG	TAAGTGCGATTGAGGGCTGT	II
YPTB2099	GGATTGAGTTTGTTGAGCAGTT	TCCGCCATCTTGTCAGTT	II

Gene	Forward primer (5'→3')	Reverse primer (5'→3')	Reference
YPTB3350	TGGCAATGTTCGTGAATTAGAG	GGCTGAATCGTTGAAAAAGG	II
YPTB1603	TGGTCAAAGGGATAAGTGTAAAAG	GCTGATGCTGTAGTGCGAAG	II
ibpA	TCTTGCTCCATTGTATCGTTC	ATGCGGTAGTTATTTTCGTCA	II
rpoE	GGCGATGTCCCTGATGTT	TCACCACGAAATGACTCCA	III
csdA	GTGATGTTGGCGAGATGGAG	ATCGTTGAGTGGGAAGCAAA	IV

4.6 Mutagenesis (II-IV) and complementation of the mutants (III, IV)

The mutants were constructed by using the TargeTron gene knockout system (Sigma-Aldrich Co.) following manufacturer's instructions. The primers used in mutagenesis and complementation are listed in Table 8. The RNA segment of the intron was retargeted, ligated into the plasmid pACD4K-C (Sigma-Aldrich Co.), and transformed to *E. coli*. Electrocompetent *Y. pseudotuberculosis* IP32953 was prepared as described previously (Conchas & Carniel, 1990), and a strain with the source of T7 RNA polymerase (pAR1219) was prepared. The pACD4K-C with the retargeted intron was introduced into the strain IP32953/pAR1219 by electroporation. Isopropyl β-D-thiogalactoside was used to induce intron expression and insertion. Insertion of the intron, the species *Y. pseudotuberculosis*, and the presence of the pYV were confirmed by PCR. Southern blotting using the PCR DIG probe synthesis kit (Roche Applied Science, Penzberg, Germany) was performed to confirm single-intron insertion in the mutant genome. Domains of genes were searched using the InterProScan tool (Hunter *et al.*, 2009).

To complement the *rpoE*475 mutant strain, the tetracycline resistance gene in pBR322 (Bolivar *et al.*, 1977) was amplified by PCR, digested, and ligated to the digested pBluescript, resulting in pBlue-*tetR*. The coding sequences of *rpoE*, *rseA*, and *rseB* and their putative native promoter were amplified by PCR. The resulting PCR product and the pBlue-*tetR* were digested and ligated, yielding pBlue-*tetR*-*rpoE*. To complement the *csdA* mutants, the coding sequence of *csdA* and its putative native promoter were amplified by PCR. The resulting PCR product and the vector pBluescript were digested and ligated, resulting in pBluescript-*csdA*. The correct sequences of the complementation plasmids were verified by sequencing. The mutants were cured of pAR1219 and confirmed by PCR for the correct species and the presence of the mutation and pYV. The complementation plasmids and the vector controls were electroporated into the mutants. The resulting strains were confirmed by PCR.

Table 8. Primers used in mutagenesis and complementation.

Primer	Sequence (5'→3')	Reference
*cheA*30-IBS	AAAAAAGCTTATAATTATCCTTAGCGTTCTATCAGGTGCGCCCAGATAGGGTG	II
*cheA*30-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCTATCAGACTAACTTACCTTTCTTTGT	II
*cheA*30-EBS2	TGAACGCAAGTTTCTAATTTCGATTAACGCTCGATAGAGGAAAGTGTCT	II
cheA243-IBS	AAAAAAGCTTATAATTATCCTTAAGTGCCCAAGCGGTGCGCCCAGATAGGGTG	II
cheA243-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCCAAGCGCATAACTTACCTTTCTTTGT	II
cheA243-EBS2	TGAACGCAAGTTTCTAATTTCGATTGCACTTCGATAGAGGAAAGTGTCT	II
cheY243-IBS	AAAAAAGCTTATAATTATCCTTATTGGCCACGTTGGTGCGCCCAGATAGGGTG	II
cheY243-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCACGTTGCCTAACTTACCTTTCTTTGT	II
cheY243-EBS2	TGAACGCAAGTTTCTAATTTCGGTTGCCAATCGATAGAGGAAAGTGTCT	II
*rpoE*475-IBS	AAAAAAGCTTATAATTATCCTTATAATGCCTGCTAGTGCGCCCAGATAGGGTG	III
*rpoE*475-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCCTGCTATCTAACTTACCTTTCTTTGT	III
*rpoE*475-EBS2	TGAACGCAAGTTTCTAATTTCGGTTCATTATCGATAGAGGAAAGTGTCT	III
*csdA*483-484-IBS	AAAAAAGCTTATAATTATCCTTACATGCCCAGCATGTGCGCCCAGATAGGGTG	IV
*csdA*483-484-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCCAGCATTTTAACTTACCTTTCTTTGT	IV
*csdA*483-484-EBS2	TGAACGCAAGTTTCTAATTTCGGTTGCATGTCGATAGAGGAAAGTGTCT	IV
*csdA*1548-1549-IBS	AAAAAAGCTTATAATTATCCTTAGTTGACGTTCGCGTGCGCCCAGATAGGGTG	IV
*csdA*1548-1549-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCGTTCGCCATAACTTACCTTTCTTTGT	IV
*csdA*1548-1549-EBS2	TGAACGCAAGTTTCTAATTTCGATTTCAACTCGATAGAGGAAAGTGTCT	IV
*csdA*1578-1579-IBS	AAAAAAGCTTATAATTATCCTTAGCTGCCGATATCGTGCGCCCAGATAGGGTG	IV

Primer	Sequence (5'→3')	Reference
*csdA*1578-1579-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCGATATCACTAACTTACCTTTCTTTGT	IV
*csdA*1578-1579-EBS2	TGAACGCAAGTTTCTAATTTCGATTGCAGCTCGATAGAGGAAAGTGTCT	IV
EBS Universal	CGAAATTAGAAACTTGCGTTCAGTAAAC	II-IV
T7	TAATACGACTCACTATAGGG	II-IV
cheA30-flank-left	GCATGGATATTACCGCGTTT	II
cheA30-flank-right	GAGCACCATCCAACAGGTTT	II
cheA243-flank-left	AAACCTGTTGGATGGTGCTC	II
cheA243-flank-right	TCGGTAGCGGATTGATTTTC	II
cheY243-flank-left	GCAGGTGGTTTCGATTTTGT	II
cheY243-flank-right	TGCTGCGATGATGTTCTCTT	II
*rpoE*475-flank-left	AGTGATGTGGATGCGAATGA	III
*rpoE*475-flank-right	CACGGAAGATACGTGAACGA	III
*csdA*483-484-flank-left	TTGTTGTTGGTACCCCAGGT	IV
*csdA*483-484-flank-right	AGAGAACAACGCGGTCTGAT	IV
csdA-flank-left	TTCTGCTGATAGCCGTGATG	IV
csdA-flank-right	CCGATATAACGGCTGCTGAT	IV

Primer	Sequence (5'→3')	Reference
N*inv*-left	TAAGGGTACTATCGCGGCGGA	(Nakajima *et al.*, 1992)
N*inv*-right	CGTGAAATTAACCGTCACACT	(Nakajima *et al.*, 1992)
K*virF*-left	TCGTGGCAGCTATGCTGTTC	(Kaneko *et al.*, 1995)
K*virF*-right	ATACGTCGCTCGCTTATCCA	(Kaneko *et al.*, 1995)
intron-left	TGGCAATGATAGCGAAACAA	II-IV
intron-right	GGTACCGCCTTGTTCACATT	II-IV
tetR-NotI	GGCGCGCGGCCGCTTCCGCGCACATTTCC	III
tetR-SacI	GGCGCGAGCTCTGTTCTGCCAAGGGTTGG	III
*rpoE*NotI	GGCGCGCGGCCGCGAGCGGGCATTGCTGG	III
*rpoE*XhoI	GGCCGCTCGAGTCATTTTGTCGACTCCAGAACCA	III
*rpoE*1	CGACTCCTGCATTAGGAAGC	III
*rpoE*2	TAGTGACTGGCGATGCTGTC	III
*rpoE*3	TGCTTGCTCAGGAAACAATG	III
*csdANot*I	GGCGCGCGGCCGCAAAAATCGGACAGAGGGCAA	IV
*csdAXho*I	GGCCGCTCGAGTTATGCATCACCGAAGCGA	IV
*csdA*1	GCTTGAAACCTCTTTTGCTGA	IV
*csdA*3	GAAAGATGGCCGTCTGGATA	IV
*csdA*5	ACGATTGAACTGCCAAAAGG	IV
RP	TTTCACACAGGAAACAGCTATGAC	III-IV

4.7 Growth experiments (II-IV) and motility tests (II)

Three separate colonies of the wild-type strain, and each mutant, complemented mutant, and vector control were grown overnight in LB broth containing ampicillin when appropriate. The overnight cultures were diluted (1:100) into fresh culture media supplemented with ampicillin, $CaCl_2$, or modified for stress experiments as appropriate. Growth was analysed in triplicate in microtitre plates in the turbidity reader Bioscreen C MBR (Oy Growth Curves Ab, Helsinki, Finland). Growth curves were generated by plotting the OD_{600} values against time. The correlation between OD_{600} values and the number of viable bacteria was confirmed by inoculating overnight culture into fresh culture media and plating on PCA supplemented with ampicillin when appropriate at several time-points during the growth.

To test the motility, the wild-type strain and the mutants were stab-inoculated into tubes containing motility test medium M103 with 2,3,5-triphenyl tetrazolium chloride (Weagant & Feng, 2001) and 0.3% agar. Bacteria were grown at 3°C, 22°C, 28°C, and 37°C, and the tubes were observed for 22, 9, 6, and 6 days, respectively.

4.8 Minimum and maximum growth temperatures (III, IV) and freezing and thawing experiments (IV)

Three separate colonies were grown overnight in LB broth containing ampicillin when appropriate. The cultures were diluted 1:100 in peptone water and stamped in triplicate using a piece of glass to cuvettes containing TSA supplemented with ampicillin when appropriate. The cuvettes were located in the Gradiplate W10 temperature gradient incubator (BCDE Group, Helsinki, Finland). For minimum growth temperature determinations, the temperature gradient was set from 1.7°C to 6.3°C or from 0.8°C to 9.5°C, and the cuvettes were incubated for 8 weeks or 10 days. For maximum growth temperature determinations, the temperature gradient was set from 40.5°C to 47.6°C or from 29.7°C to 44.6°C for 2 days. The Nixon SMZ-U stereomicroscope (Nikon, Tokyo, Japan) was used to determine the limits of growth. Statistical significance of the differences between growth temperatures was evaluated with Student's t test.

To investigate the freeze-thaw tolerance of the *csdA* mutant strains and the wild-type strain, 1.5 ml of overnight culture of each strain were transferred into a cryovial in duplicate and frozen at -20°C. Strains were thawed and re-frozen 15 times at 24-h intervals and plated on PCA to determine colony-forming units/ml at 72-h intervals. Student's t test was used to evaluate differences between the survival rate of the wild-type strain and the mutants.

5 RESULTS

5.1 Sequence variability of virulence genes (I)

Investigation of sequence variability of virulence genes *inv*, *virF*, and *yadA* of 18 *Y. pseudotuberculosis* strains revealed the largest sequence variability in *yadA* and the smallest in *virF*. The amino acid sequences of the *inv* genes varied at most by 1.5%. The two *Y. similis* strains investigated had a frame-shift mutation due to a 1-bp deletion in the *inv* sequence that caused truncation of the signal peptide from 48 amino acids to 31 amino acids. The sequence of *virF* of the *Y. pseudotuberculosis* strains did not vary at the amino acid level. The nucleotide and amino acid sequences of *yadA* varied at most 2.2% and 5.2%, respectively, between the *Y. pseudotuberculosis* strains. Sequence variability was concentrated in the N-terminus of YadA. Thus, most of the nucleotide substitutions altered the amino acid. According to amino acid sequences of *yadA*, the strains could be divided into nine groups.

5.2 Role of two-component systems in cold tolerance (II)

The relative expression levels of 54 genes predicted to encode TCSs in *Y. pseudotuberculosis* IP32953 were determined at 28°C and at 3°C using RT-qPCR. Results were normalized to 16S *rrn*, the expression of which was constant at 3°C and 28°C. The expression of 44 genes was significantly higher ($p<0.05$) at 3°C than at 28°C. TCS CheA/CheY encoding genes *cheA* and *cheY* had the highest relative expression levels at 3°C. Their relative expression was 31- and 25-fold higher, respectively, than their expression at 28°C. In the majority of the TCSs, all genes of the system had significantly higher expression at 3°C than at 28°C. *YPTB2099* was the only gene that had a lower relative expression level at 3°C than at 28°C, and the UhpB/UhpA TCS was the only one from the predicted 24 complete TCSs in the strain IP32953 that had constant expression at 3°C and 28°C.

Three insertional mutations were constructed to further study the role of the CheA/CheY TCS. The group II intron was inserted in sense orientation in the mutants *cheA*30 and *cheY*243 and in antisense orientation in the mutant *cheA*243. At 3°C, growth of the mutants *cheA*243 and *cheA*30 was impaired, while growth of the mutant *cheY*243 was comparable with that of the wild-type strain. In motility tests, all mutants were non-motile at 3°C and at 22°C, while the wild-type strain had umbrella-type motility at 3°C and 22°C. All strains were non-motile at 28°C and at 37°C.

5.3 Role of alternative sigma factor σ^E in stress tolerance (III)

RT-qPCR was used to determine the relative expression levels of *rpoE* encoding σ^E under temperature, pH, osmotic, and ethanol stress. Stably expressed 16S *rrn* was used in the normalization. Relative expression of *rpoE* was significantly higher under low and high temperature, acid and alkaline, osmotic, and ethanol stress in the early logarithmic growth phase than expression under optimal growth conditions in the same growth phase. In the late logarithmic growth phase, *rpoE* expression was induced by heat stress.

The *rpoE*475 mutant was constructed, and growth was investigated at 3°C, 28°C, 37°C, and 42°C and at 28°C at pH 5.0, pH 9.0, 3% NaCl, and 3% ethanol. At 3°C, the growth of the mutant ceased in the late logarithmic growth phase (Fig. 1A). There was no difference in the growth of the complemented mutant and wild-type, and the vector control and the mutant. At 28°C, the mutant and wild-type had similar growths (Fig. 1B). At 37°C, the lag phase of the mutant was longer than that of wild-type (Fig. 1C). Lag phase was shortened but growth rate was decreased in the complemented mutant relative to the wild-type. The vector control had impaired growth. At 42°C, the mutant did not attain OD_{600} values similar to the wild-type strain (Fig. 1D). The complemented mutant reached OD_{600} values as high as the wild-type, but the growth rate was slower. Growth of the vector control was abolished. At pH 5.0, the lag phase of the mutant was longer than the lag phase of the wild-type strain (Fig. 2A). The complemented mutant had a similar lag phase as the wild-type, but it grew slower than the wild-type, and growth of the vector control was impaired. At pH 9.0 and under osmotic stress in 3% NaCl, the mutant and the wild-type had comparable growths (Fig. 2B-C). At 3% ethanol, the mutant and the vector control did not grow at all (Fig. 2D). The complemented mutant grew at a slower rate than the wild-type strain, and OD_{600} values similar to the wild-type were not attained. OD_{600} values and the number of viable bacteria correlated at 28°C and under all stress conditions investigated.

The minimum and maximum growth temperatures of the *rpoE*475 mutant were 1.2°C and 36.1°C, respectively, while those of wild-type were under 0.8°C and 43.5°C, respectively. The changes were statistically significant. The minimum growth temperature of the complemented mutant was 1.3°C, and that of the vector control 2.5°C. The complemented mutant and the vector control had maximum growth temperatures of 41.3°C and 31.7°C, respectively.

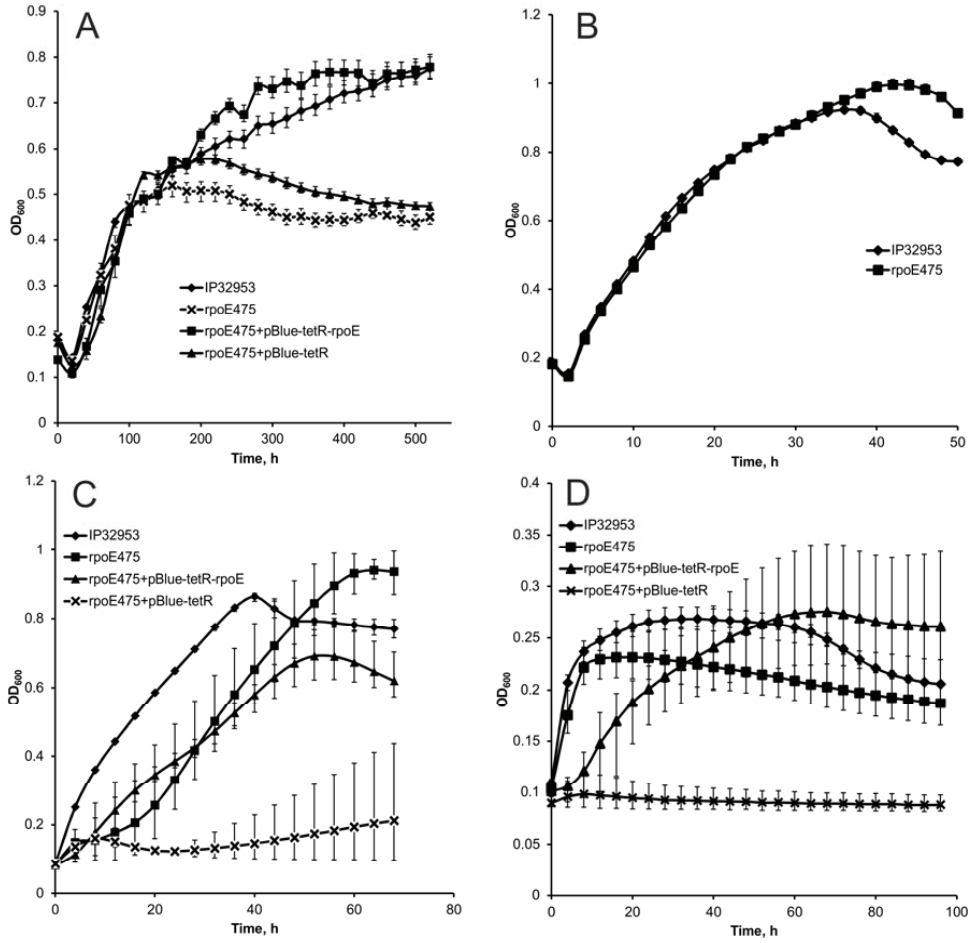

Figure 1 Growth curves of the *Yersinia pseudotuberculosis* IP32953 wild-type strain, *rpoE*475 mutant strain, complemented mutant, and vector control at 3°C (A), 28°C (B), 37°C (C), and 42°C (D). Measured OD_{600} values are shown at 20-h intervals (A), at 2-h intervals (B), and at 4-h intervals (C and D). Error bars indicate minimum and maximum values. Figure derived from Study III.

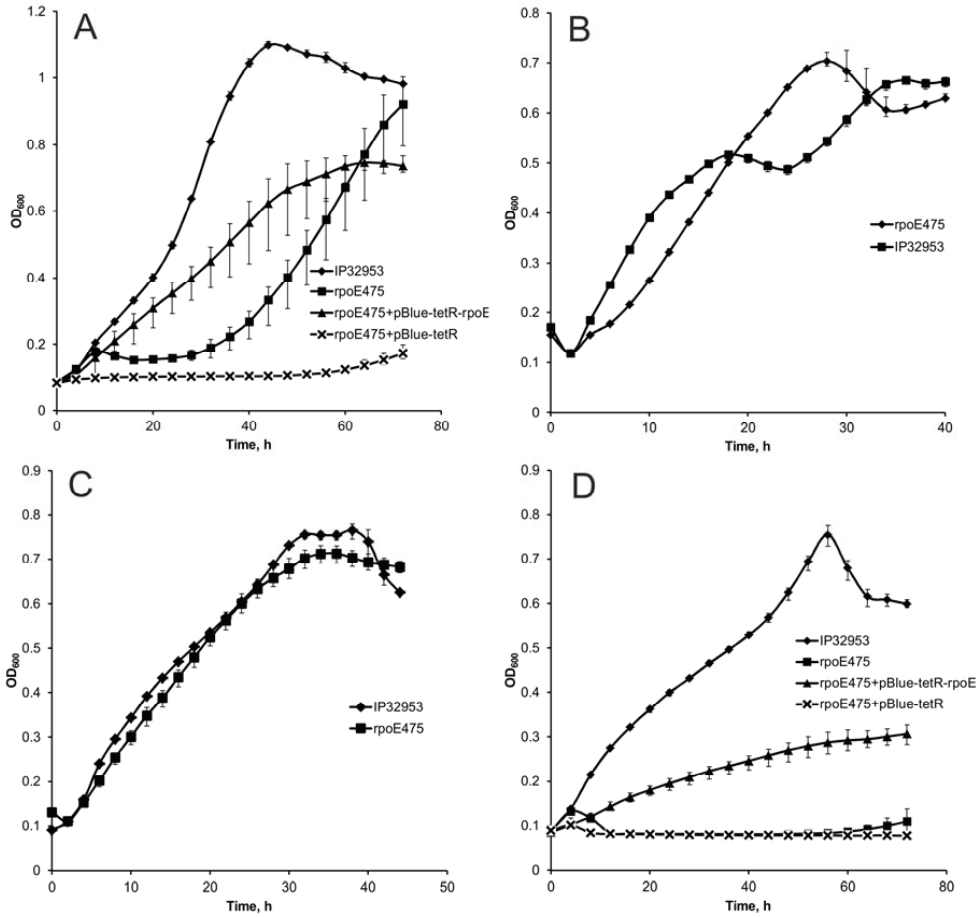

Figure 2 Growth curves of the *Yersinia pseudotuberculosis* IP32953 wild-type strain, *rpoE*475 mutant strain, complemented mutant, and vector control at pH 5.0 (A), pH 9.0 (B), 3% NaCl (C), and 3% ethanol (D). Measured OD_{600} values are shown at 4-h intervals (A and D) and at 2-h intervals (B and C). Error bars indicate minimum and maximum values. Figure derived from Study III.

5.4 Role of DEAD-box RNA helicase CsdA in cold tolerance (IV)

Relative expression levels of *csdA* at 3°C and at 28°C in the early logarithmic growth phase were determined by RT-qPCR. At 3°C, the relative expression level of *csdA* was 9.4-fold higher than at 28°C. Insertional mutants *csdA*483, *csdA*1548, and *csdA*1578 were constructed to further investigate the role of CsdA. The mutant *csdA*483 had the intron targeted in antisense orientation to the DEAD/DEAH-box helicase domain (PF00270), and *csdA*1548 and *csdA*1578 mutants had the intron targeted to the DbpA RNA binding domain (PF03880) in sense and in antisense orientation, respectively. During mutagenesis the pYV was lost from *csdA*483, and despite several attemps, the pYV was not kept in *csdA*483. The other mutants, *csdA*1548 and *csdA*1578, did however, retain the pYV.

Growth of the mutants and the wild-type strain was investigated at 3°C and 28°C. The mutant *csdA*483 did not grow at 3°C, and growth of the mutants *csdA*1548 and *csdA*1578 at 3°C was severely impaired (Fig. 3A). The OD_{600} values of the wild-type and the mutants correlated with the number of viable bacteria. Growth of none of the mutants at 28°C differed from the wild-type strain (Fig. 3B). When a complementation plasmid pBluescript-*csdA* containing the coding sequence of *csdA* and its putative promoter was introduced into the mutants, growth was better than the growth of the vector controls at 3°C (Fig. 3C-E). The growth of the vector controls did not differ from the growth of the mutants. The complementation plasmid did not affect the growth of the wild-type strain (Fig. 3F).

In the freezing and thawing experiments, the average number of viable cells of the wild-type, *csdA*1548, and *csdA*1578 strains declined from 10^8 to 10^2, and that of the *csdA*483 declined from 10^8 to 10^1. However, the changes were not significantly different from those of the wild-type strain. In the minimum growth temperature experiment, the strains IP32953, *csdA*1548, and *csdA*1578 grew over the temperature gradient (1.7-6.3°C), meaning that the minimum growth temperatures of these strains are lower than 1.7°C. The minimum growth temperature of *csdA*483 (5.6°C) was significantly higher than that of the wild-type strain. In the maximum growth temperature experiment, no significant differences emerged between *csdA*483 (43.7°C), *csdA*1548 (43.2°C), *csdA*1578 (43.2°C), and the wild-type strain (43.0°C).

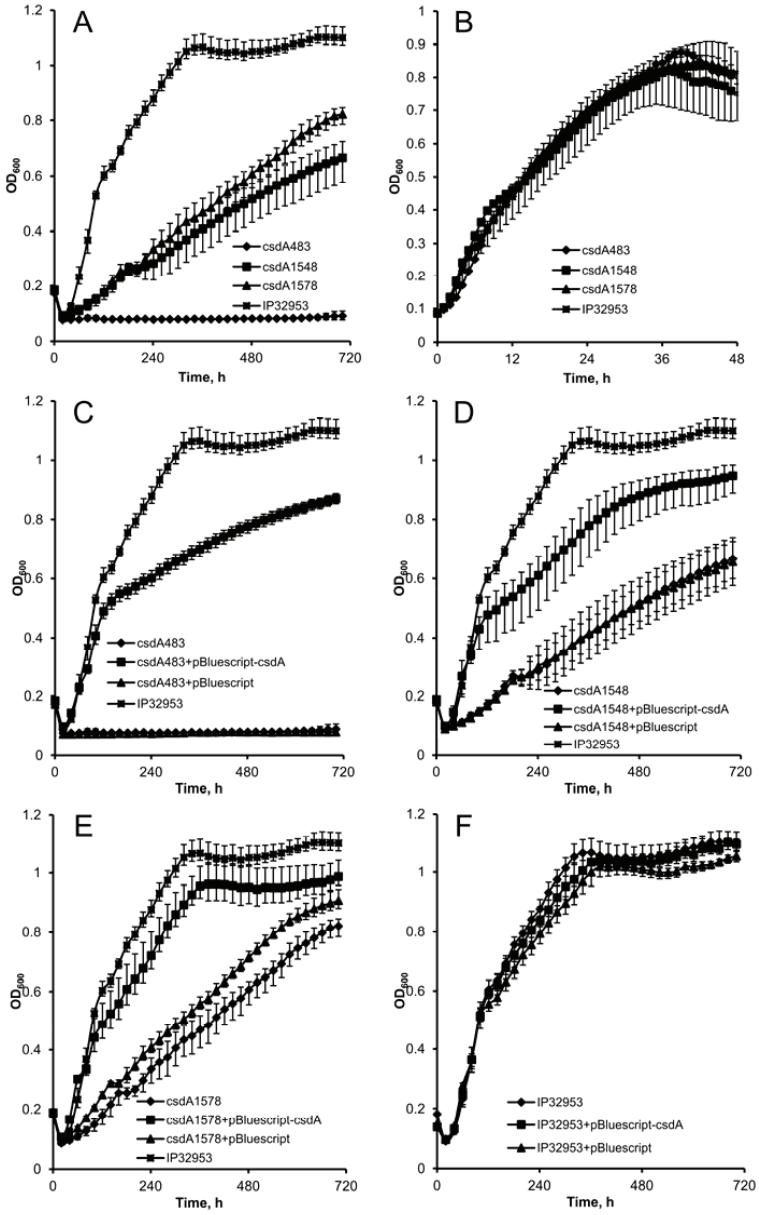

Figure 3 Growth curves of the *Yersinia pseudotuberculosis* IP32953 wild-type strain and *csdA* mutants at 3°C (A) and at 28°C (B). Growth of the mutants, complemented mutants, vector-only controls, and the wild-type strain at 3°C (C-F). In graphs (A) and (C-F), measured values are shown at 20-h intervals, in (B), at 1-h intervals. Error bars indicate minimum and maximum values. Figure adapted from Fig. 2 in Study IV.

6 DISCUSSION

6.1 Genetic variability is limited in *inv* and *virF* sequences and considerable in *yadA* sequences (I)

The virulence genes *inv* and *virF* are highly similar in the *Y. pseudotuberculosis* strains investigated. Thus, PCR with the commonly used primers targeted to *inv* (Kageyama *et al.*, 2002, Kaneko *et al.*, 1995, Nakajima *et al.*, 1992, Thoerner *et al.*, 2003) and *virF* (Harnett *et al.*, 1996, Kageyama *et al.*, 2002, Kaneko *et al.*, 1995, Lambertz & Danielsson-Tham, 2005, Thoerner *et al.*, 2003, Wren & Tabaqchali, 1990) detect the bacterium well. However, PCR using the primers targeted to *inv* can also detect some *Y. similis* strains. The considerable variability of *yadA* can hinder detection by PCR, and detection primers should be targeted to conserved areas of *yadA*. In addition, the variability of *yadA* was concentrated at the top of the YadA head. These changes can influence the functional properties of YadA. Previous reports have demonstrated that variation of YadA may affect the virulence of *Y. pseudotuberculosis* (Heise & Dersch, 2006). Moreover, effects on the immune responses of the host are possible, as established in *C. botulinum*, where 7% variability at the amino acid level of the neurotoxin gene sequence affected antigenic properties of botulinum neurotoxin (Lou *et al.*, 2010).

6.2 Two-component system encoding genes are induced at 3°C and two-component system CheA/CheY is essential for optimal growth at 3°C (II)

Relative expression levels of genes encoding 24 complete TCSs and 5 orphan hydrid histidine kinases or response regulators of the *Y. pseudotuberculosis* strain IP32953 were determined at 3°C and at 28°C using RT-qPCR. The stable expression of 16S *rrn* at 3°C and 28°C confirmed 16S *rrn* as a suitable reference gene for studies conducted at low temperatures on this pathogen. Most genes had higher relative expression levels at 3°C than at 28°C. Several TCS encoding genes have been reported to be induced at low temperatures also in *E. coli* and *L. monocytogenes* when studied by DNA microarrays (Chan *et al.*, 2007, Moen *et al.*, 2009, White-Ziegler *et al.*, 2008). Some TCSs of *Yersinia* have previously been linked to stress tolerance, but the significant induction of TCS encoding genes at cold temperatures has not been demonstrated earlier in *Y. pseudotuberculosis*.

Genes encoding chemotaxis proteins CheA and CheY had clearly the highest relative expression levels at 3°C. In previous studies on *Y. enterocolitica* and *E. coli*, expression of *cheA* and *cheY* has been induced at low temperatures (Bresolin *et al.*, 2006, Phadtare & Inouye, 2004). The role of CheA and CheY under cold stress was studied further by insertional mutagenesis. Growth of the histidine kinase CheA mutants *cheA*30 and *cheA*243 was impaired at 3°C, while growth of the response regulator mutant *cheY*243 did

not differ from that of the wild-type strain at 3°C. Thus, CheY may not be essential for optimal growth at low temperatures or, alternatively, the mutation site near the C-terminus of the *cheY* affected the results. Growth of the mutants and the wild-type strain was similar at 28°C. Based on the impaired growth of the *cheA* mutants at low temperatures, CheA probably functions as a sensor for low temperature. Chemotaxis proteins are members of the flagellar regulon (Soutourina & Bertin, 2003), and their mutation should result in a non-motile phenotype. Expectedly, the *cheA* and *cheY* mutants were non-motile at all temperatures tested. Also non-motile *L. monocytogenes* mutants have reduced cold tolerance (Markkula *et al.*, 2012, Mattila *et al.*, 2011). Thus, it appears that motility is required for optimal growth at low temperatures.

In six TCSs, expression of only one of the genes encoding the system was induced at 3°C, indicating possible inactivation of some components and re-established cross-talk between non-cognate histidine kinases and response regulators. Approximately one-third of the histidine kinases and response regulators of *E. coli* can phosphorylate or be phosphorylated, respectively, by non-cognate response regulators and histidine kinases *in vitro* (Yamamoto *et al.*, 2005), but *in vivo* cross-talk is rare (Groban *et al.*, 2009, Podgornaia & Laub, 2013). Cells can utilize cross-regulation, i.e. advantageous cross-talk, in situations where combining multiple signals into one response or expanding one signal to many responses is beneficial (Laub & Goulian, 2007). Whether cross-regulation is used at low temperatures in *Y. pseudotuberculosis* warrants further investigation. However, the significant induction of TCS encoding genes at low temperatures in *Y. pseudotuberculosis* indicates that TCSs are probably members of complex signalling networks that enable the growth of this pathogen at low temperatures.

6.3 σ^E is required for stress tolerance (III)

This study demonstrated upregulation of rpoE expression by low or high temperature, acidic or alkaline pH, increased osmolality, and 3% ethanol by RT-qPCR. The majority of the upregulated transcription occurred in the early logarithmic growth phase. Since several genes of the σ^E regulon have a role in the synthesis of outer membrane components and are especially needed during active cell division (Rhodius *et al.*, 2006), this result was expected. In *E. coli*, σ^E-dependent envelope stress response is induced by several stresses disturbing composition or folding of cell envelope proteins such as heat (Erickson & Gross, 1989), increased expression of outer membrane proteins (Mecsas *et al.*, 1993), ethanol (Raina *et al.*, 1995), or high osmolality (Bianchi & Baneyx, 1999). Thus, the induced *rpoE* expression in *Y. pseudotuberculosis* by temperature, pH, osmotic, and ethanol stress is consistent with *E. coli*.

Mutational analysis was used to further investigate the role of σ^E. Impaired or abolished growth of the *rpoE*475 mutant at pH 5.0, at 3°C, at 37°C, at 42°C, and at 3% ethanol demonstrated that functional σ^E is essential under several stress conditions in *Y. pseudotuberculosis*. In addition, the minimum and maximum growth temperatures of the *rpoE*475 mutant (1.2°C and 36.1°C) were higher and lower, respectively, than those of the wild-type strain (<0.8°C and 43.5°C). In *Y. enterocolitica*, expression of *rpoE* is induced

in Peyer's patches at the beginning of infection in mice (Heusipp *et al.*, 2003, Young & Miller, 1997). In *Y. pseudotuberculosis*, σ^E has a role in the proper function of the Ysc-Yop type III secretion system (Carlsson *et al.*, 2007a). In *Enterobacteriaceae*, several genes associated with pathogenesis belong to the σ^E regulon (Rhodius *et al.*, 2006), and σ^E is pivotal to survival in the host and full virulence (Raivio, 2005). The significant decrease in the maximum growth temperature of the *rpoE*475 mutant below the mammalian body temperature confirms the role of σ^E in virulence in *Y. pseudotuberculosis*. In addition, the essential role of σ^E under stress conditions encountered in the food chain was demonstrated.

6.4 CsdA is essential for growth at low temperatures (IV)

This study demonstrated that insertion of the intron into *csdA* either abolished or impaired growth at 3°C, indicating that CsdA is essential for growth of *Y. pseudotuberculosis* IP32953 at low temperatures. Growth of the *csdA*483 was totally suppressed, and *csdA*1548 and *csdA*1578 had clearly impaired growth at 3°C. Previous reports have demonstrated an indispensable role for CsdA in optimal growth at 25°C or temperatures below this in *E. coli* (Awano *et al.*, 2007, Charollais *et al.*, 2004, Jones *et al.*, 1996, Turner *et al.*, 2007), and at 10°C in *B. cereus* (Broussolle *et al.*, 2010, Pandiani *et al.*, 2010).

In *Y. pseudotuberculosis* IP32953, CsdA seems not to have a role in freeze-thaw tolerance. Also in *L. monocytogenes*, mutations of RNA helicase genes did not affect freeze-thaw tolerance (Azizoglu & Kathariou, 2010, Markkula *et al.*, 2012). The observed rise in minimum growth temperature of the *csdA*483 mutant is also in line with *L. monocytogenes*, where RNA helicase mutants had significantly higher minimum growth temperatures than the wild-type strain (Markkula *et al.*, 2012). Thus, *csdA* in *Y. pseudotuberculosis* seems to be important during continuous growth at cold temperatures. In *E. coli*, another RNA helicase, RhlE, can compensate for the absence of *csdA* when overexpressed in the cells of a plasmid (Awano *et al.*, 2007, Jain, 2008). Hence the main role of CsdA at low temperatures is supposed to be due to its helicase function (Awano *et al.*, 2007, Turner *et al.*, 2007). In *E. coli*, CsdA participates in mRNA decay at low temperatures by destabilizing mRNAs, enabling their degradation by RNases and PNPase (Awano *et al.*, 2007, Yamanaka & Inouye, 2001). It has been demonstrated in *Y. enterocolitica* that until cold-shock protein mRNAs are degraded after cold shock, cells do not continue to grow (Neuhaus *et al.*, 2000). Whether CsdA plays a critical role in mRNA degradation at low temperatures in *Y. pseudotuberculosis* warrants further investigations.

6.5 Stress tolerance of *Yersinia pseudotuberculosis* (II-IV)

The means that bacteria use to adapt to stressful conditions include changes in their gene expression by utilizing specific sigma factors or TCSs and synthesis of specific proteins such as enzymes and cold-shock proteins. *Y. pseudotuberculosis* has a large growth temperature range (Bottone *et al.*, 2005, Fredriksson-Ahomaa *et al.*, 2010). It is able to survive for long periods in the environment (Jalava *et al.*, 2006, Rimhanen-Finne *et al.*, 2009), and it tolerates a wide pH range (Bottone *et al.*, 2005) and also high osmolality (Fredriksson-Ahomaa *et al.*, 2010). By using TCSs and σ^E, *Y. pseudotuberculosis* can rapidly change its gene expression pattern under stress conditions, and synthesis of specific proteins, such as cold-induced helicases, can begin. The observations of Studies II-IV indicate that *Y. pseudotuberculosis* exploits several mechanisms in adapting to environmental stress. Stress tolerance mechanisms overlap, as shown by the important role of σ^E under several stress conditions, and probably also affect virulence. However, effects of the TCSs, σ^E, and cold-induced proteins on virulence of *Y. pseudotuberculosis* warrant further investigation.

7 CONCLUSIONS

1. Genetic variability of *inv* and *virF* is limited between different *Y. pseudotuberculosis* strains, and PCR with primers targeted to these genes detect the bacterium well. In contrast, *yadA* varies considerably between different strains, and detection primers should target conserved areas of *yadA*.

2. Expression of several genes encoding TCSs in *Y. pseudotuberculosis* IP32953 is induced at 3°C, and genes encoding TCS CheA/CheY had the highest relative expression levels at 3°C. In addition, optimal growth at 3°C requires *cheA*, and both *cheA* and *cheY* are needed for motility in this pathogen.

3. Mutational analysis revealed that functional σ^E is essential under acid, cold, heat, and ethanol stress in *Y. pseudotuberculosis* IP32953. Furthermore, mutation of *rpoE* encoding σ^E narrows the growth temperature range of the pathogen. Results indicate an important role for σ^E in stress tolerance, and thus, survival of *Y. pseudotuberculosis* IP32953 in the food chain.

4. Mutation of *csdA* abolished or impaired growth at 3°C. Also the minimum growth temperature of the *csdA* mutant was higher than that of the wild-type strain. CsdA is essential for cold tolerance of *Y. pseudotuberculosis* IP32953.

8 REFERENCES

Abe J, Takeda T, Watanabe Y, Nakao H, Kobayashi N, Leung DY, & Kohsaka T (1993) Evidence for superantigen production by *Yersinia pseudotuberculosis*. *J Immunol* **151**: 4183-4188

Achtman M, Zurth K, Morelli G, Torrea G, Guiyoule A, & Carniel E (1999) *Yersinia pestis*, the cause of plague, is a recently emerged clone of *Yersinia pseudotuberculosis*. *Proc Natl Acad Sci U S A* **96**: 14043-14048

Agafonov DE, Kolb VA, Nazimov IV, & Spirin AS (1999) A protein residing at the subunit interface of the bacterial ribosome. *Proc Natl Acad Sci U S A* **96**: 12345-12349

Agafonov DE & Spirin AS (2004) The ribosome-associated inhibitor A reduces translation errors. *Biochem Biophys Res Commun* **320**: 354-358

Aguilar PS, Cronan Jr JE, & de Mendoza D (1998) A *Bacillus subtilis* gene induced by cold shock encodes a membrane phospholipid desaturase. *J Bacteriol* **180**: 2194-2200

Aguilar PS, Hernandez-Arriaga AM, Cybulski LE, Erazo AC, & De Mendoza D (2001) Molecular basis of thermosensing: a two-component signal transduction thermometer in *Bacillus subtilis*. *EMBO J* **20**: 1681-1691

Albrecht H (1910) Zur Aetiologie der Enteritis follicularis suppurativa. *Wien klin Wschr* **23**: 991-994

Allen KJ, Lepp D, McKellar RC, & Griffiths MW (2008) Examination of stress and virulence gene expression in *Escherichia coli* O157:H7 using targeted microarray analysis. *Foodborne Pathog Dis* **5**: 437-447

Atkinson S, Chang CY, Patrick HL, Buckley CMF, Wang Y, Sockett RE, Cámara M, & Williams P (2008) Functional interplay between the *Yersinia pseudotuberculosis* YpsRI and YtbRI quorum sensing systems modulates swimming motility by controlling expression of *flhDC* and *fliA*. *Mol Microbiol* **69**: 137-151

Atlung T & Hansen FG (1999) Low-temperature-induced DnaA protein synthesis does not change initiation mass in *Escherichia coli* K-12. *J Bacteriol* **181**: 5557-5562

Aulisio CC, Mehlman IJ, & Sanders AC (1980) Alkali method for rapid recovery of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* from foods. *Appl Environ Microbiol* **39**: 135-140

Awano N, Xu C, Ke H, Inoue K, Inouye M, & Phadtare S (2007) Complementation analysis of the cold-sensitive phenotype of the *Escherichia coli csdA* deletion strain. *J Bacteriol* **189**: 5808-5815

Azizoglu RO & Kathariou S (2010) Inactivation of a cold-induced putative RNA helicase gene of *Listeria monocytogenes* is accompanied by failure to grow at low temperatures but does not affect freeze-thaw tolerance. *J Food Prot* **73**: 1474-1479

Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, & Mori H (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol* **2**: 2006.0008

Badger JL & Miller VL (1995) Role of RpoS in survival of *Yersinia enterocolitica* to a variety of environmental stresses. *J Bacteriol* **177**: 5370-5373

Bakholdina SI, Sanina NM, Krasikova IN, Popova OB, & Solov'eva TF (2004) The impact of abiotic factors (temperature and glucose) on physicochemical properties of lipids from *Yersinia pseudotuberculosis*. *Biochimie* **86**: 875-881

Battesti A, Majdalani N, & Gottesman S (2011) The RpoS-mediated general stress response in *Escherichia coli*. *Annu Rev Microbiol* **65**: 189-213

Bergann T, Kleemann J, & Sohr D (1995) Modeluntersuchungen zur Psychrotrophie von *Yersinia enterocolitica*. *J Vet Med Ser B* **42**: 523-531

Bergman T, Erickson K, Galyov E, Persson C, & Wolf-Watz H (1994) The *lcrB* (*yscN/U*) gene cluster of *Yersinia pseudotuberculosis* is involved in Yop secretion and shows high homology to the spa gene clusters of *Shigella flexneri* and *Salmonella typhimurium*. *J Bacteriol* **176**: 2619-2626

Bianchi AA & Baneyx F (1999) Hyperosmotic shock induces the σ^{32} and σ^{E} stress regulons of *Escherichia coli*. *Mol Microbiol* **34**: 1029-1038

Biedzka-Sarek M, Venho R, & Skurnik M (2005) Role of YadA, Ail, and lipopolysaccharide in serum resistance of *Yersinia enterocolitica* serotype O:3. *Infect Immun* **73**: 2232-2244

Blattner FR, Plunkett G,3rd, Bloch CA, Perna NT, Burland V, Riley M, Collado-Vides J, Glasner JD, Rode CK, Mayhew GF, Gregor J, Davis NW, Kirkpatrick HA, Goeden MA, Rose DJ, Mau B, & Shao Y (1997) The complete genome sequence of *Escherichia coli* K-12. *Science* **277**: 1453-1462

Bliska JB, Copass MC, & Falkow S (1993) The *Yersinia pseudotuberculosis* adhesin YadA mediates intimate bacterial attachment to and entry into HEp-2 cells. *Infect Immun* **61**: 3914-3921

Böhme K, Steinmann R, Kortmann J, Seekircher S, Heroven AK, Berger E, Pisano F, Thiermann T, Wolf-Watz H, & Narberhaus F (2012) Concerted actions of a thermolabile regulator and a unique intergenic RNA thermosensor control *Yersinia* virulence. *PLoS Pathog* **8**: e1002518

Bolivar F, Rodriguez RL, Greene PJ, Betlach MC, Heyneker HL, Boyer HW, Crosa JH, & Falkow S (1977) Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* **2**: 95-113

Bottone EJ, Bercovier H, Mollaret HH (2005) Genus XLI. *Yersinia*. In *Bergey's Manual of Systematic Bacteriology,* Garrity GM, Brenner DJ, Krieg NR, Staley JR (eds) pp 838-848. Springer-Verlag: East Lansing, MI, USA

Bozue J, Mou S, Moody KL, Cote CK, Trevino S, Fritz D, & Worsham P (2011) The role of the *phoPQ* operon in the pathogenesis of the fully virulent CO92 strain of *Yersinia pestis* and the IP32953 strain of *Yersinia pseudotuberculosis*. *Microb Pathog* **50**: 314-321

Brandi A, Spurio R, Gualerzi CO, & Pon CL (1999) Massive presence of the *Escherichia coli* 'major cold-shock protein' CspA under non-stress conditions. *EMBO J* **18**: 1653-1659

Bresolin G, Trcek J, Scherer S, & Fuchs TM (2008) Presence of a functional flagellar cluster Flag-2 and low-temperature expression of flagellar genes in *Yersinia enterocolitica* W22703. *Microbiology* **154**: 196-206

Bresolin G, Neuhaus K, Scherer S, & Fuchs TM (2006) Transcriptional analysis of long-term adaptation of *Yersinia enterocolitica* to low-temperature growth. *J Bacteriol* **188**: 2945-2958

Broussolle V, Pandiani F, Haddad N, Michaud C, Carlin F, Nguyen-the C, & Brillard J (2010) Insertional mutagenesis reveals genes involved in *Bacillus cereus* ATCC 14579 growth at low temperature. *FEMS Microbiol Lett* **306**: 177-183

Buck M, Gallegos M, Studholme DJ, Guo Y, & Gralla JD (2000) The bacterial enhancer-dependent σ^{54} (σ^{N}) transcription factor. *J Bacteriol* **182**: 4129-4136

Buhles W, Vanderlip J, Russell S, & Alexander N (1981) *Yersinia pseudotuberculosis* infection: study of an epizootic in squirrel monkeys. *J Clin Microbiol* **13**: 519-525

Capra EJ & Laub MT (2012) Evolution of two-component signal transduction systems. *Annu Rev Microbiol* **66**: 325-347

Carlsson KE, Liu J, Edqvist PJ, & Francis MS (2007a) Extracytoplasmic-stress-responsive pathways modulate type III secretion in *Yersinia pseudotuberculosis*. *Infect Immun* **75**: 3913-3924

Carlsson KE, Liu J, Edqvist PJ, & Francis MS (2007b) Influence of the Cpx extracytoplasmic-stress-responsive pathway on *Yersinia sp*.-eukaryotic cell contact. *Infect Immun* **75**: 4386-4399

Carniel E, Autenrieth I, Cornelis G, Fukushima H, Guinet F, Isberg R, Pham J, Prentice M, Simonet M, Skurnik M, Wauters G (2006) *Y. enterocolitica* and *Y. pseudotuberculosis*. In *The Prokaryotes, A Handbook on the Biology of Bacteria, Volume 6: Proteobacteria: Gamma Subclass,* Dworkin M, Falkow S, Rosenberg E, Schleifer K-, Stackebrandt E (eds) pp 270-398. Springer: New York

Carniel E (2001) The *Yersinia* high-pathogenicity island: an iron-uptake island. *Microb Infect* **3**: 561-569

Carniel E (1999) The *Yersinia* high-pathogenicity island. *Int Microbiol* **2**: 161-167

Carniel E, Mazigh D, & Mollaret HH (1987) Expression of iron-regulated proteins in *Yersinia* species and their relation to virulence. *Infect Immun* **55**: 277-280

Carniel E, Mercereau-Puijalon O, & Bonnefoy S (1989) The gene coding for the 190,000-dalton iron-regulated protein of *Yersinia* species is present only in the highly pathogenic strains. *Infect Immun* **57**: 1211-1217

Carnoy C, Müller-Alouf H, Desreumaux P, Mullet C, Grangette C, & Simonet M (2000) The superantigenic toxin of *Yersinia pseudotuberculosis*: a novel virulence factor? *Int J Med Microbiol* **290**: 477-482

Carnoy C, Floquet S, Marceau M, Sebbane F, Haentjens-Herwegh S, Devalckenaere A, & Simonet M (2002) The superantigen gene *ypm* is located in an unstable chromosomal locus of *Yersinia pseudotuberculosis*. *J Bacteriol* **184**: 4489-4499

Chain PSG, Carniel E, Larimer FW, Lamerdin J, Stoutland PO, Regala WM, Georgescu AM, Vergez LM, Land ML, & Motin VL (2004) Insights into the evolution of *Yersinia pestis* through whole-genome comparison with *Yersinia pseudotuberculosis*. *Proc Natl Acad Sci U S A* **101**: 13826-13831

Champion OL, Cooper IA, James SL, Ford D, Karlyshev A, Wren BW, Duffield M, Oyston PC, & Titball RW (2009) *Galleria mellonella* as an alternative infection model for *Yersinia pseudotuberculosis*. *Microbiology* **155**: 1516-1522

Chan YC, Raengpradub S, Boor KJ, & Wiedmann M (2007) Microarray-based characterization of the *Listeria monocytogenes* cold regulon in log- and stationary-phase cells. *Appl Environ Microbiol* **73**: 6484-6498

Chan YC, Hu Y, Chaturongakul S, Files KD, Bowen BM, Boor KJ, & Wiedmann M (2008) Contributions of two-component regulatory systems, alternative factors, and negative regulators to *Listeria monocytogenes* cold adaptation and cold growth. *J Food Prot* **71**: 420-425

Charollais J, Dreyfus M, & Iost I (2004) CsdA, a cold-shock RNA helicase from *Escherichia coli*, is involved in the biogenesis of 50S ribosomal subunit. *Nucleic Acids Res* **32**: 2751-2759

Cohen-Or I, Shenhar Y, Biran D, & Ron EZ (2010) CspC regulates *rpoS* transcript levels and complements *hfq* deletions. *Res Microbiol* **161**: 694-700

Conchas RF & Carniel E (1990) A highly efficient electroporation system for transformation of *Yersinia*. *Gene* **87**: 133-137

Cork S, Marshall R, Madie P, & Fenwick S (1995) The role of wild birds and the environment in the epidemiology of *Yersiniae* in New Zealand. *N Z Vet J* **43**: 169-174

Cornelis GR, Sluiters C, Delor I, Geib D, Kaniga K, Rouvroit CL, Sory MP, Vanooteghem JC, & Michiels T (1991) *ymoA*, a *Yersinia enterocolitica* chromosomal gene modulating the expression of virulence functions. *Mol Microbiol* **5**: 1023-1034

Cornelis GR (2006) The type III secretion injectisome. *Nat Rev Microbiol* **4**: 811-825

Cornelis GR & Wolf-Watz H (1997) The *Yersinia* Yop virulon: a bacterial system for subverting eukaryotic cells. *Mol Microbiol* **23**: 861-867

Cornelis G, Sluiters C, de Rouvroit CL, & Michiels T (1989) Homology between VirF, the transcriptional activator of the *Yersinia* virulence regulon, and AraC, the *Escherichia coli* arabinose operon regulator. *J Bacteriol* **171**: 254-262

Dahlsten E, Kirk D, Lindström M, & Korkeala H (2013) Alternative sigma factor SigK has a role in stress tolerance of Group I *Clostridium botulinum* strain ATCC 3502. *Appl Environ Microbiol* **79**: 3867-3869

de Almeida AM, Guiyoule A, Guilvout I, Iteman I, Baranton G, & Carniel E (1993) Chromosomal *irp2* gene in *Yersinia*: distribution, expression, deletion and impact on virulence. *Microb Pathog* **14**: 9-21

De Castro C, Skurnik M, Molinaro A, & Holst O (2009) Characterization of the specific O-polysaccharide structure and biosynthetic gene cluster of *Yersinia pseudotuberculosis* serotype O:15. *Innate Immun* **15**: 351-359

De Koning-Ward TF & Robins-Browne RM (1995) Contribution of urease to acid tolerance in *Yersinia enterocolitica*. *Infect Immun* **63**: 3790-3795

De Las Penas A, Connolly L, & Gross CA (1997) σ^E is an essential sigma factor in *Escherichia coli*. *J Bacteriol* **179**: 6862-6864

Derman Y, Isokallio M, Lindström M, & Korkeala H (2013) The two-component system CBO2306/CBO2307 is important for cold adaptation of *Clostridium botulinum* ATCC 3502. *Int J Food Microbiol* **167**: 87-91

Dhar MS, Gupta V, & Virdi JS (2013) Detection, distribution and characterization of novel superoxide dismutases from *Yersinia enterocolitica* biovar 1A. *PLoS ONE* **8**: e63919

Ding L, Wang Y, Hu Y, Atkinson S, Williams P, & Chen S (2009) Functional characterization of FlgM in the regulation of flagellar synthesis and motility in *Yersinia pseudotuberculosis*. *Microbiology* **155**: 1890-1900

Eitel J, Heise T, Thiesen U, & Dersch P (2005) Cell invasion and IL-8 production pathways initiated by YadA of *Yersinia pseudotuberculosis* require common signalling molecules (FAK, c-Src, Ras) and distinct cell factors. *Cell Microbiol* **7**: 63-77

Eitel J & Dersch P (2002) The YadA protein of *Yersinia pseudotuberculosis* mediates high-efficiency uptake into human cells under environmental conditions in which invasin is repressed. *Infect Immun* **70**: 4880-4891

Erickson JW & Gross CA (1989) Identification of the σ^E subunit of *Escherichia coli* RNA polymerase: a second alternate σ factor involved in high-temperature gene expression. *Genes Dev* **3**: 1462-1471

European Food Safety Authority (2012) The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in the European Union in 2010. *EFSA Journal* **10**: 190-205

Ewing B & Green P (1998) Base-calling of automated sequencer traces using *Phred*. II. Error probabilities. *Genome Res* **8**: 186-194

Fisher ML, Castillo C, & Mecsas J (2007) Intranasal inoculation of mice with *Yersinia pseudotuberculosis* causes a lethal lung infection that is dependent on *Yersinia* outer proteins and PhoP. *Infect Immun* **75**: 429-442

Flamez C, Ricard I, Arafah S, Simonet M, & Marceau M (2008) Phenotypic analysis of *Yersinia pseudotuberculosis* 32777 response regulator mutants: New insights into two-component system regulon plasticity in bacteria. *Int J Med Microbiol* **298**: 193-207

Fredriksson-Ahomaa M & Korkeala H (2003) Low occurrence of pathogenic *Yersinia enterocolitica* in clinical, food, and environmental samples: a methodological problem. *Clin Microbiol Rev* **16**: 220-229

Fredriksson-Ahomaa M, Lindström M, Korkeala H (2010) *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*. In *Pathogens and Toxins in Foods: Challenges and*

Interventions, Juneja VK & Sofos JN (eds) pp 164-180. ASM Press: Washington, D.C., USA

Fredriksson-Ahomaa M, Wacheck S, Koenig M, Stolle A, & Stephan R (2009) Prevalence of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* in wild boars in Switzerland. *Int J Food Microbiol* **135**: 199-202

Fukushima H, Matsuda Y, Seki R, Tsubokura M, Takeda N, Shubin FN, Paik IK, & Zheng XB (2001) Geographical heterogeneity between Far Eastern and Western countries in prevalence of the virulence plasmid, the superantigen *Yersinia pseudotuberculosis*-derived mitogen, and the high-pathogenicity island among *Yersinia pseudotuberculosis* strains. *J Clin Microbiol* **39**: 3541-3547

Fukushima H, Gomyoda M, & Kaneko S (1990) Mice and moles inhabiting mountainous areas of Shimane Peninsula as sources of infection with *Yersinia pseudotuberculosis*. *J Clin Microbiol* **28**: 2448-2455

Fukushima H, Nakamura R, Iitsuka S, Ito Y, & Saito K (1985) Presence of zoonotic pathogens (Yersinia spp., *Campylobacter jejuni*, *Salmonella spp*., and *Leptospira spp*.) simultaneously in dogs and cats. *Zbl Bakt Hyg I Abt Orig B* **181**: 430-440

Fukushima H & Gomyoda M (1991) Intestinal carriage of *Yersinia pseudotuberculosis* by wild birds and mammals in Japan. *Appl Environ Microbiol* **57**: 1152-1155

Fukushima H, Gomyoda M, Ishikura S, Nishio T, Moriki S, Endo J, Kaneko S, & Tsubokura M (1989) Cat-contaminated environmental substances lead to *Yersinia pseudotuberculosis* infection in children. *J Clin Microbiol* **27**: 2706-2709

Fukushima H, Gomyoda M, Shiozawa K, Kaneko S, & Tsubokura M (1988) *Yersinia pseudotuberculosis* infection contracted through water contaminated by a wild animal. *J Clin Microbiol* **26**: 584-585

Gemski P, Lazere JR, Casey T, & Wohlhieter JA (1980) Presence of a virulence-associated plasmid in *Yersinia pseudotuberculosis*. *Infect Immun* **28**: 1044-1047

Giangrossi M, Brandi A, Giuliodori AM, Gualerzi CO, & Pon CL (2007) Cold-shock-induced *de novo* transcription and translation of *infA* and role of IF1 during cold adaptation. *Mol Microbiol* **64**: 807-821

Gibson JL, Lombardo MJ, Thornton PC, Hu KH, Galhardo RS, Beadle B, Habib A, Magner DB, Frost LS, Herman C, Hastings PJ, & Rosenberg SM (2010) The σ^E stress response is required for stress-induced mutation and amplification in *Escherichia coli*. *Mol Microbiol* **77**: 415-430

Giuliodori AM, Brandi A, Giangrossi M, Gualerzi CO, & Pon CL (2007) Cold-stress-induced *de novo* expression of *infC* and role of IF3 in cold-shock translational bias. *RNA* **13**: 1355-1365

Giuliodori AM, Brandi A, Gualerzi CO, & Pon CL (2004) Preferential translation of cold-shock mRNAs during cold adaptation. *RNA* **10**: 265-276

Goldenberg D, Azar I, Oppenheim A, Brandi A, Pon C, & Gualerzi C (1997) Role of *Escherichia coli cspA* promoter sequences and adaptation of translational apparatus in the cold shock response. *Mol Gen Genet* **256**: 282-290

Goldenberg D, Azar I, & Oppenheim AB (1996) Differential mRNA stability of the *cspA* gene in the cold-shock response of *Escherichia coli*. *Mol Microbiol* **19**: 241-248

Göpel Y, Lüttmann D, Heroven AK, Reichenbach B, Dersch P, & Görke B (2011) Common and divergent features in transcriptional control of the homologous small RNAs GlmY and GlmZ in *Enterobacteriaceae*. *Nucleic Acids Res* **39**: 1294-1309

Goverde RLJ, Huis in't Veld JHJ, Kusters JG, & Mooi FR (1998) The psychrotrophic bacterium *Yersinia enterocolitica* requires expression of *pnp*, the gene for polynucleotide phosphorylase, for growth at low temperature (5^{o}C). *Mol Microbiol* **28**: 555

Goverde RLJ & Kusters JG (1994) Growth rate and physiology of *Yersinia enterocolitica*; influence of temperature and presence of the virulence plasmid. *J Appl Bacteriol* **77**: 96-104

Grabenstein JP, Marceau M, Pujol C, Simonet M, & Bliska JB (2004) The response regulator PhoP of *Yersinia pseudotuberculosis* is important for replication in macrophages and for virulence. *Infect Immun* **72**: 4973-4984

Grassl GA, Bohn E, Müller Y, Bühler OT, & Autenrieth IB (2003) Interaction of *Yersinia enterocolitica* with epithelial cells: invasin beyond invasion. *Int J Med Microbiol* **293**: 41-54

Graumann PL & Marahiel MA (1998) A superfamily of proteins that contain the cold-shock domain. *Trends Biochem Sci* **23**: 286-290

Groban ES, Clarke EJ, Salis HM, Miller SM, & Voigt CA (2009) Kinetic buffering of cross talk between bacterial two-component sensors. *J Mol Biol* **390**: 380-393

Gruber TM & Gross CA (2003) Multiple sigma subunits and the partitioning of bacterial transcription space. *Annu Rev Microbiol* **57**: 441-466

Gualerzi CO, Giuliodori AM, & Pon CL (2003) Transcriptional and post-transcriptional control of cold-shock genes. *J Mol Biol* **331**: 527-539

Gueguen E, Durand E, Zhang XY, d'Amalric Q, Journet L, & Cascales E (2013) Expression of a *Yersinia pseudotuberculosis* type VI secretion system is responsive to envelope stresses through the OmpR transcriptional activator. *PLoS ONE* **8**: e66615

Harnett N, Lin Y, & Krishnan C (1996) Detection of pathogenic *Yersinia enterocolitica* using the multiplex polymerase chain reaction. *Epidemiol Infect* **117**: 59-68

Heise T & Dersch P (2006) Identification of a domain in *Yersinia* virulence factor YadA that is crucial for extracellular matrix-specific cell adhesion and uptake. *Proc Natl Acad Sci U S A* **103**: 3375-3380

Hengge R (2009) Proteolysis of σ^S (RpoS) and the general stress response in *Escherichia coli*. *Res Microbiol* **160**: 667-676

Hengge-Aronis R (2002) Signal transduction and regulatory mechanisms involved in control of the σ^S (RpoS) subunit of RNA polymerase. *Microbiol Mol Biol Rev* **66**: 373-395

Heroven AK, Böhme K, Rohde M, & Dersch P (2008) A Csr-type regulatory system, including small non-coding RNAs, regulates the global virulence regulator RovA of *Yersinia pseudotuberculosis* through RovM. *Mol Microbiol* **68**: 1179-1195

Heroven AK, Böhme K, Dersch P (2012a) Regulation of virulence gene expression by regulatory RNA elements in *Yersinia pseudotuberculosis*. In *Advances in Yersinia Research,* Anonymous pp 315-323. Springer: New York

Heroven AK, Sest M, Pisano F, Scheb-Wetzel M, Steinmann R, Böhme K, Klein J, Münch R, Schomburg D, & Dersch P (2012b) Crp induces switching of the CsrB and CsrC RNAs in *Yersinia pseudotuberculosis* and links nutritional status to virulence. *Front Cell Infect Microbiol* **2**

Heusipp G, Schmidt MA, & Miller VL (2003) Identification of *rpoE* and *nadB* as host responsive elements of *Yersinia enterocolitica*. *FEMS Microbiol Lett* **226**: 291-298

Hinchliffe SJ, Howard SL, Huang YH, Clarke DJ, & Wren BW (2008) The importance of the Rcs phosphorelay in the survival and pathogenesis of the enteropathogenic yersiniae. *Microbiology* **154**: 1117-1131

Ho DK, Riva R, Kirjavainen V, Jarva H, Ginstrom E, Blom AM, Skurnik M, & Meri S (2012a) Functional recruitment of the human complement inhibitor C4BP to *Yersinia pseudotuberculosis* outer membrane protein Ail. *J Immunol* **188**: 4450-4459

Ho DK, Riva R, Skurnik M, & Meri S (2012b) The *Yersinia pseudotuberculosis* outer membrane protein Ail recruits the human complement regulatory protein factor H. *J Immunol* **189**: 3593-3599

Hoiczyk E, Roggenkamp A, Reichenbecher M, Lupas A, & Heesemann J (2000) Structure and sequence analysis of *Yersinia* YadA and *Moraxella* UspAs reveal a novel class of adhesins. *EMBO J* **19**: 5989-5999

Horne SM & Prüß BM (2006) Global gene regulation in *Yersinia enterocolitica*: effect of FliA on the expression levels of flagellar and plasmid-encoded virulence genes. *Arch Microbiol* **185**: 115-126

Hu Y, Lu P, Wang Y, Ding L, Atkinson S, & Chen S (2009a) OmpR positively regulates urease expression to enhance acid survival of *Yersinia pseudotuberculosis*. *Microbiology* **155**: 2522-2531

Hu Y, Wang Y, Ding L, Lu P, Atkinson S, & Chen S (2009b) Positive regulation of *flhDC* expression by OmpR in *Yersinia pseudotuberculosis*. *Microbiology* **155**: 3622-3631

Hunter S, Apweiler R, Attwood TK, Bairoch A, Bateman A, Binns D, Bork P, Das U, Daugherty L, & Duquenne L (2009) InterPro: the integrative protein signature database. *Nucleic Acids Res* **37**: D211-D215

Hurst MR, Becher SA, Young SD, Nelson TL, & Glare TR (2011) *Yersinia entomophaga* sp. nov., isolated from the New Zealand grass grub *Costelytra zealandica*. *Int J Syst Evol Microbiol* **61**: 844-849

Imlay JA & Linn S (1988) DNA damage and oxygen radical toxicity. *Science* **240**: 1302-1309

Iriarte M, Stainier I, Mikulskis AV, & Cornelis GR (1995a) The *fliA* gene encoding σ^{28} in *Yersinia enterocolitica*. *J Bacteriol* **177**: 2299-2304

Iriarte M, Stainier I, & Cornelis GR (1995b) The *rpoS* gene from *Yersinia enterocolitica* and its influence on expression of virulence factors. *Infect Immun* **63**: 1840-1847

Isberg RR, Swain A, & Falkow S (1988) Analysis of expression and thermoregulation of the *Yersinia pseudotuberculosis inv* gene with hybrid proteins. *Infect Immun* **56**: 2133-2138

Isberg RR, Voorhis DL, & Falkow S (1987) Identification of invasin: a protein that allows enteric bacteria to penetrate cultured mammalian cells. *Cell* **50**: 769-778

Jaakola S, Lyytikäinen O, Rimhanen-Finne R, Salmenlinna S, Vuopio J, Roivainen M, Löflund J, Kuusi M, Ruutu P (eds) (2012) *Infectious diseases in Finland 2011.* National Institute for Health and Welfare (THL): Tampere, Finland

Jain C (2008) The *E. coli* RhlE RNA helicase regulates the function of related RNA helicases during ribosome assembly. *RNA* **14**: 381-389

Jalava K, Hakkinen M, Valkonen M, Nakari UM, Palo T, Hallanvuo S, Ollgren J, Siitonen A, & Nuorti JP (2006) An outbreak of gastrointestinal illness and erythema nodosum from grated carrots contaminated with *Yersinia pseudotuberculosis*. *J Infect Dis* **194**: 1209-1216

Jalava K, Hallanvuo S, Nakari UM, Ruutu P, Kela E, Heinasmaki T, Siitonen A, & Nuorti JP (2004) Multiple outbreaks of *Yersinia pseudotuberculosis* infections in Finland. *J Clin Microbiol* **42**: 2789-2791

Jerrett I, Slee K, & Robertson B (1990) Yersiniosis in farmed deer. *Aust Vet J* **67**: 212-214

Jiang W, Hou Y, & Inouye M (1997) CspA, the major cold-shock protein of *Escherichia coli*, is an RNA chaperone. *J Biol Chem* **272**: 196-202

Jones PG & Inouye M (1996) RbfA, 30s ribosomal binding factor, is cold-shock protein whose absence triggers the cold-shock response. *Mol Microbiol* **21**: 1207-1218

Jones PG, Mitta M, Kim Y, Jiang W, & Inouye M (1996) Cold shock induces a major ribosomal-associated protein that unwinds double-stranded RNA in *Escherichia coli*. *Proc Natl Acad Sci U S A* **93**: 76-80

Jones PG, VanBogelen RA, & Neidhardt FC (1987) Induction of proteins in response to low temperature in *Escherichia coli*. *J Bacteriol* **169**: 2092-2095

Kageyama T, Ogasawara A, Fukuhara R, Narita Y, Miwa N, Kamanaka Y, Abe M, Kumazaki K, Maeda N, & Suzuki J (2002) *Yersinia pseudotuberculosis* infection in breeding monkeys: detection and analysis of strain diversity by PCR. *J Med Primatol* **31**: 129-135

Kandror O, DeLeon A, & Goldberg AL (2002) Trehalose synthesis is induced upon exposure of *Escherichia coli* to cold and is essential for viability at low temperatures. *Proc Natl Acad Sci U S A* **99**: 9727

Kaneko S, Ishizaki N, & Kokubo Y (1995) Detection of pathogenic *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* from pork using the polymerase chain reaction. *Contr Microbiol Immunol* **13**: 153-155

Kangas S, Takkinen J, Hakkinen M, Nakari UM, Johansson T, Henttonen H, Virtaluoto L, Siitonen A, Ollgren J, & Kuusi M (2008) *Yersinia pseudotuberculosis* O:1 traced to raw carrots, Finland. *Emerg Infect Dis* **14**: 1959-1961

Kanjee U & Houry WA (2013) Mechanisms of acid resistance in *Escherichia coli*. *Annu Rev Microbiol* **67**: 65-81

Kannan K, Janiyani KL, Shivaji S, & Ray MK (1998) Histidine utilisation operon (*hut*) is upregulated at low temperature in the antarctic psychrotrophic bacterium *Pseudomonas syringae*. *FEMS Microbiol Lett* **161**: 7-14

Kapatral V & Minnich SA (1995) Co-ordinate, temperature-sensitive regulation of the three *Yersinia enterocolitica* flagellin genes. *Mol Microbiol* **17**: 49-56

Kapatral V, Olson JW, Pepe JC, Miller VL, & Minnich SA (1996) Temperature-dependent regulation of *Yersinia enterocolitica* Class III flagellar genes. *Mol Microbiol* **19**: 1061-1071

Karlyshev AV, Oyston PCF, Williams K, Clark GC, Titball RW, Winzeler EA, & Wren BW (2001) Application of high-density array-based signature-tagged mutagenesis to discover novel *Yersinia* virulence-associated genes. *Infect Immun* **69**: 7810-7819

Khemici V, Toesca I, Poljak L, Vanzo NF, & Carpousis AJ (2004) The RNase E of *Escherichia coli* has at least two binding sites for DEAD-box RNA helicases: functional replacement of RhlB by RhlE. *Mol Microbiol* **54**: 1422-1430

Knapp W (1954) *Pasteurella pseudotuberculosis* als Erreger einer mesenterialen Lymphadenitis beim Menschen. *Zbl Bakt Hyg I Abt Orig* **161**: 422-424

Knapp W & Masshoff W (1954) Zur Ätiologie der abszedierenden retikulozytären Lymphadenitis. *Dtsch med Wschr* **79**: 1266-1271

La Teana A, Brandi A, Falconi M, Spurio R, & Pon CL (1991) Identification of a cold shock transcriptional enhancer of the *Escherichia coli* gene encoding nucleoid protein H-NS. *Proc Natl Acad Sci U S A* **88**: 10907-10911

Lambertz ST & Danielsson-Tham ML (2005) Identification and characterization of pathogenic *Yersinia enterocolitica* isolates by PCR and pulsed-field gel electrophoresis. *Appl Environ Microbiol* **71**: 3674-3681

Lãtnada EB, Morris RS, Jackson R, & Fenwick SG (2005) A cohort study of *Yersinia* infection in goats. *Aust Vet J* **83**: 567-571

Laub MT & Goulian M (2007) Specificity in two-component signal transduction pathways. *Annu Rev Genet* **41**: 121-145

Laukkanen R, Martinez PO, Siekkinen KM, Ranta J, Maijala R, & Korkeala H (2008) Transmission of *Yersinia pseudotuberculosis* in the pork production chain from farm to slaughterhouse. *Appl Environ Microbiol* **74**: 5444-5450

Laukkanen R, Niskanen T, Fredriksson-Ahomaa M, & Korkeala H (2003) *Yersinia pseudotuberculosis* in pigs and pig houses in Finland. *Adv Exp Med Biol* **529**: 371-373

Laukkanen-Ninios R, Didelot X, Jolley KA, Morelli G, Sangal V, Kristo P, Brehony C, Imori PFM, Fukushima H, Siitonen A, Tseneva G, Voskressenskaya E, Falcao JP, Korkeala H, Maiden MCJ, Mazzoni C, Carniel E, Skurnik M, & Achtman M (2011)

Population structure of the *Yersinia pseudotuberculosis* complex according to multilocus sequence typing. *Environ Microbiol*

Leo JC & Skurnik M (2011) Adhesins of human pathogens from the genus *Yersinia*. *Adv Exp Med Biol* **715**: 1-15

Linder P, Lasko PF, Leroy P, Nielsen PJ, Nishi K, Schnier J, & Slonimski PP (1989) Birth of the D-E-A-D box. *Nature* **337**: 121-122

Lindström M, Dahlsten E, Soderholm H, Selby K, Somervuo P, Heap JT, Minton NP, & Korkeala H (2012) Involvement of two-component system CBO0366/CBO0365 in the cold shock response and growth of group I (proteolytic) *Clostridium botulinum* ATCC 3502 at low temperatures. *Appl Environ Microbiol* **78**: 5466-5470

Liu J, Obi IR, Thanikkal EJ, Kieselbach T, & Francis MS (2011) Phosphorylated CpxR restricts production of the RovA global regulator in *Yersinia pseudotuberculosis*. *PLoS ONE* **6**: e23314

Liu J, Thanikkal EJ, Obi IR, & Francis MS (2012) Elevated CpxR~P levels repress the Ysc-Yop type III secretion system of *Yersinia pseudotuberculosis*. *Res Microbiol* **163**: 518-530

Lou J, Geren I, Garcia-Rodriguez C, Forsyth C, Wen W, Knopp K, Brown J, Smith T, Smith L, & Marks J (2010) Affinity maturation of human botulinum neurotoxin antibodies by light chain shuffling via yeast mating. *Protein Eng Des Sel* **23**: 311-319

Lu J, Aoki H, & Ganoza MC (1999) Molecular characterization of a prokaryotic translation factor homologous to the eukaryotic initiation factor eIF4A. *Int J Biochem Cell Biol* **31**: 215-229

Madrid C, Nieto JM, & Juárez A (2001) Role of the Hha/YmoA family of proteins in the thermoregulation of the expression of virulence factors. *Int J Med Microbiol* **291**: 425-432

Malassez L & Vignal W (1884) Sur le micro-organisme de la tuberculose zoogloéique. *Arch Physiol Norm Pathol* **3**: 81-105

Maldonado-Arocho FJ, Green C, Fisher ML, Paczosa MK, & Mecsas J (2013) Adhesins and host serum factors drive Yop translocation by *Yersinia* into professional phagocytes during animal infection. *PLoS Pathog* **9**: e1003415

Mansilla MC, Cybulski LE, Albanesi D, & de Mendoza D (2004) Control of membrane lipid fluidity by molecular thermosensors. *J Bacteriol* **186**: 6681-6688

Marceau M (2005) Transcriptional regulation in *Yersinia*: an update. *Curr Issues Mol Biol* **7**: 151-178

Marceau M, Sebbane F, Ewann F, Collyn F, Lindner B, Campos MA, Bengoechea JA, & Simonet M (2004) The *pmrF* polymyxin-resistance operon of *Yersinia pseudotuberculosis* is upregulated by the PhoP-PhoQ two-component system but not by PmrA-PmrB, and is not required for virulence. *Microbiology* **150**: 3947-3957

Markkula A, Mattila M, Lindström M, & Korkeala H (2012) Genes encoding putative DEAD-box RNA helicases in *Listeria monocytogenes* EGD-e are needed for growth and motility at 3° C. *Environ Microbiol* **14**: 2223-2232

Marra A & Isberg RR (1997) Invasin-dependent and invasin-independent pathways for translocation of *Yersinia pseudotuberculosis* across the Peyer's patch intestinal epithelium. *Infect Immun* **65**: 3412-3421

Martinez PO, Fredriksson-Ahomaa M, Sokolova Y, Roasto M, Berzins A, & Korkeala H (2009) Prevalence of enteropathogenic *Yersinia* in Estonian, Latvian, and Russian (Leningrad region) pigs. *Foodborne Pathog Dis* **6**: 719-724

Mascher G, Derman Y, Kirk DG, Palonen E, Lindström M, & Korkeala H (2014) The CLO3403/CLO3404 two-component system of *Clostridium botulinum* E1 Beluga is important for cold shock response and growth at low temperatures. *Appl Environ Microbiol* **80**: 399-407

Masshoff W & Dölle W (1953) Über eine besondere Form der sog. mesenterialen Lymphadenopathie: "Die abscedierende reticulocytäre Lymphadenitis". *Virchows Arch path Anat* **323**: 664-684

Mattila M, Lindström M, Somervuo P, Markkula A, & Korkeala H (2011) Role of *flhA* and *motA* in growth of *Listeria monocytogenes* at low temperatures. *Int J Food Microbiol* **148**: 177-183

Mattila M, Somervuo P, Rattei T, Korkeala H, Stephan R, & Tasara T (2012) Phenotypic and transcriptomic analyses of sigma L-dependent characteristics in *Listeria monocytogenes* EGD-e. *Food Microbiol* **32**: 152-164

Mecsas J, Rouviere PE, Erickson JW, Donohue T, & Gross CA (1993) The activity of σ^E, an *Escherichia coli* heat-inducible σ-factor, is modulated by expression of outer membrane proteins. *Genes Dev* **7**: 2618-2628

Mejía E, Bliska JB, & Viboud GI (2008) *Yersinia* controls type III effector delivery into host cells by modulating Rho activity. *PLoS Pathog* **4**: e3

Merhej V, Adékambi T, Pagnier I, Raoult D, & Drancourt M (2008) *Yersinia massiliensis* sp. nov., isolated from fresh water. *Int J Syst Evol Microbiol* **58**: 779-784

Meysick KC, Seidman J, & Falconio JR (2009) The *Yersinia pseudotuberculosis* YplA phospholipase differs in its activity, regulation and secretion from that of the *Yersinia enterocolitica* YplA. *Microb Pathog* **47**: 24-32

Michiels T, Vanooteghem JC, Lambert de Rouvroit C, China B, Gustin A, Boudry P, & Cornelis GR (1991) Analysis of *virC*, an operon involved in the secretion of Yop proteins by *Yersinia enterocolitica*. *J Bacteriol* **173**: 4994-5009

Miller VL & Falkow S (1988) Evidence for two genetic loci in *Yersinia enterocolitica* that can promote invasion of epithelial cells. *Infect Immun* **56**: 1242-1248

Mitta M, Fang L, & Inouye M (1997) Deletion analysis of *cspA* of *Escherichia coli*: Requirement of the AT-rich UP element for *cspA* transcription and the downstream box in the coding region for its cold shock induction. *Mol Microbiol* **26**: 321-335

Miyoshi-Akiyama T, Imanishi K, & Uchiyama T (1993) Purification and partial characterization of a product from *Yersinia pseudotuberculosis* with the ability to activate human T cells. *Infect Immun* **61**: 3922-3927

Moen B, Janbu AO, Langsrud S, Langsrud O, Hobman JL, Constantinidou C, Kohler A, & Rudi K (2009) Global responses of *Escherichia coli* to adverse conditions determined by microarrays and FT-IR spectroscopy. *Can J Microbiol* **55**: 714-728

Mollaret HH (1995) Fifteen centuries of Yersiniosis. *Contrib Microbiol Immunol* **13**: 1-4

Mulder B, Michiels T, Simonet M, Sory MP, & Cornelis G (1989) Identification of additional virulence determinants on the pYV plasmid of *Yersinia enterocolitica* W227. *Infect Immun* **57**: 2534-2541

Murros-Kontiainen A, Johansson P, Niskanen T, Fredriksson-Ahomaa M, Korkeala H, & Björkroth J (2011a) *Yersinia pekkanenii* sp. nov. *Int J Syst Evol Microbiol* **61**: 2363-2367

Murros-Kontiainen A, Fredriksson-Ahomaa M, Korkeala H, Johansson P, Rahkila R, & Björkroth J (2011b) *Yersinia nurmii* sp. nov. *Int J Syst Evol Microbiol* **61**: 2368-2372

Na J, Im H, & Lee K (2011) Expression and purification of recombinant superoxide dismutase (PaSOD) from *Psychromonas arctica* in *Escherichia coli*. *Bull Korean Chem Soc* **32**: 2405-2409

Nagamachi E, Shibuya S, Hirai Y, Matsushita O, Tomochika K, & Kanemasa Y (1991) Adaptational changes of fatty acid composition and the physical state of membrane lipids following the change of growth temperature in *Yersinia enterocolitica*. *Microbiol Immunol* **35**: 1085-1093

Nagel G, Lahrz A, & Dersch P (2001) Environmental control of invasin expression in *Yersinia pseudotuberculosis* is mediated by regulation of RovA, a transcriptional activator of the SlyA/Hor family. *Mol Microbiol* **41**: 1249-1269

Nakajima H, Inoue M, Mori T, Itoh K, Arakawa E, & Watanabe H (1992) Detection and identification of *Yersinia pseudotuberculosis* and pathogenic *Yersinia enterocolitica* by an improved polymerase chain reaction method. *J Clin Microbiol* **30**: 2484-2486

Netterling S, Vaitkevicius K, Nord S, & Johansson J (2012) A *Listeria monocytogenes* RNA helicase essential for growth and ribosomal maturation at low temperatures uses its C terminus for appropriate interaction with the ribosome. *J Bacteriol* **194**: 4377-4385

Neuhaus K, Francis KP, Rapposch S, Görg A, & Scherer S (1999) Pathogenic *Yersinia* species carry a novel, cold-inducible major cold shock protein tandem gene duplication producing both bicistronic and monocistronic mRNA. *J Bacteriol* **181**: 6449

Neuhaus K, Anastasov N, Kaberdin V, Francis KP, Miller VL, & Scherer S (2003) The AGUAAA motif in *cspA1/A2* mRNA is important for adaptation of *Yersinia enterocolitica* to grow at low temperature. *Mol Microbiol* **50**: 1629-1645

Neuhaus K, Rapposch S, Francis KP, & Scherer S (2000) Restart of exponential growth of cold-shocked *Yersinia enterocolitica* occurs after down-regulation of *cspA1/A2* mRNA. *J Bacteriol* **182**: 3285-3288

Ninfa AJ & Magasanik B (1986) Covalent modification of the *glnG* product, NR_I, by the *glnL* product, NR_{II}, regulates the transcription of the *glnALG* operon in *Escherichia coli*. *Proc Natl Acad Sci U S A* **83**: 5909-5913

Niskanen T, Laukkanen R, Fredriksson-Ahomaa M, & Korkeala H (2008) Distribution of *virF/lcrF*-positive *Yersinia pseudotuberculosis* serotype O:3 at farm level. *Zoonoses Public Health* **55**: 214-221

Niskanen T, Laukkanen R, Murros A, Björkroth J, Skurnik M, Korkeala H, & Fredriksson-Ahomaa M (2009) Characterisation of non-pathogenic *Yersinia pseudotuberculosis*-like strains isolated from food and environmental samples. *Int J Food Microbiol* **129**: 150-156

Niskanen T, Fredriksson-Ahomaa M, & Korkeala H (2002) *Yersinia pseudotuberculosis* with limited genetic diversity is a common finding in tonsils of fattening pigs. *J Food Prot* **65**: 540-545

Nixon BT, Ronson CW, & Ausubel FM (1986) Two-component regulatory systems responsive to environmental stimuli share strongly conserved domains with the nitrogen assimilation regulatory genes *ntrB* and *ntrC*. *Proc Natl Acad Sci U S A* **83**: 7850-7854

Nowgesic E, Fyfe M, Hockin J, King A, Ng H, Paccagnella A, Trinidad A, Wilcott L, Smith R, Denney A, Struck L, Embree G, Higo K, Chan JI, Markey P, Martin S, & Bush D (1999) Outbreak of *Yersinia pseudotuberculosis* in British Columbia - November 1998. *Can Commun Dis Rep* **25**: 97-100

Nuorti JP, Niskanen T, Hallanvuo S, Mikkola J, Kela E, Hatakka M, Fredriksson-Ahomaa M, Lyytikainen O, Siitonen A, Korkeala H, & Ruutu P (2004) A widespread outbreak of *Yersinia pseudotuberculosis* O:3 infection from iceberg lettuce. *J Infect Dis* **189**: 766-774

Okwori AEJ, Martínez PO, Fredriksson-Ahomaa M, Agina SE, & Korkeala H (2009) Pathogenic *Yersinia enterocolitica* 2/O: 9 and *Yersinia pseudotuberculosis* 1/O: 1 strains isolated from human and non-human sources in the Plateau State of Nigeria. *Food Microbiol* **26**: 872-875

Ortiz Martínez P, Mylona S, Drake I, Fredriksson-Ahomaa M, Korkeala H, & Corry JEL (2010) Wide variety of bioserotypes of enteropathogenic *Yersinia* in tonsils of English pigs at slaughter. *Int J Food Microbiol* **139**: 64-69

Österberg S, Peso-Santos Td, & Shingler V (2011) Regulation of alternative sigma factor use. *Annu Rev Microbiol* **65**: 37-55

Paczosa MK, Fisher ML, Maldonado-Arocho FJ, & Mecsas J (2014) *Yersinia pseudotuberculosis* uses Ail and YadA to circumvent neutrophils by directing Yop translocation during lung infection. *Cell Microbiol* **16**: 247-268

Palonen E, Lindström M, & Korkeala H (2010) Adaptation of enteropathogenic *Yersinia* to low growth temperature. *Crit Rev Microbiol* **36**: 54-67

Pandiani F, Brillard J, Bornard I, Michaud C, Chamot S, Nguyen-the C, & Broussolle V (2010) Differential involvement of the five RNA helicases in adaptation of *Bacillus cereus* ATCC 14579 to low growth temperatures. *Appl Environ Microbiol* **76**: 6692-6697

Peil L, Virumäe K, & Remme J (2008) Ribosome assembly in *Escherichia coli* strains lacking the RNA helicase DeaD/CsdA or DbpA. *FEBS Journal* **275**: 3772-3782

Pepe JC, Badger JL, & Miller VL (1994) Growth phase and low pH affect the thermal regulation of the *Yersinia enterocolitica inv* gene. *Mol Microbiol* **11**: 123-135

Pettersson J, Nordfelth R, Dubinina E, Bergman T, Gustafsson M, Magnusson KE, & Wolf-Watz H (1996) Modulation of virulence factor expression by pathogen target cell contact. *Science* **273**: 1231-1233

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Res* **29**: 2002-2007

Phadtare S & Inouye M (2004) Genome-wide transcriptional analysis of the cold shock response in wild-type and cold-sensitive, quadruple-*csp*-deletion strains of *Escherichia coli*. *J Bacteriol* **186**: 7007-7014

Phadtare S (2004) Recent developments in bacterial cold-shock response. *Curr Issues Mol Biol* **6**: 125-136

Phadtare S & Inouye M (2001) Role of CspC and CspE in regulation of expression of RpoS and UspA, the stress response proteins in *Escherichia coli*. *J Bacteriol* **183**: 1205-1214

Pitcher DG, Saunders NA, & Owen RJ (1989) Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Lett Appl Microbiol* **8**: 151-156

Podgornaia AI & Laub MT (2013) Determinants of specificity in two-component signal transduction. *Curr Opin Microbiol* **16**: 156-162

Polissi A, De Laurentis W, Zangrossi S, Briani F, Longhi V, Pesole G, & Deho G (2003) Changes in *Escherichia coli* transcriptome during acclimatization at low temperature. *Res Microbiol* **154**: 573-580

Prud'homme-Généreux A, Beran RK, Iost I, Ramey CS, Mackie GA, & Simons RW (2004) Physical and functional interactions among RNase E, polynucleotide phosphorylase and the cold-shock protein, CsdA: evidence for a 'cold shock degradosome'. *Mol Microbiol* **54**: 1409-1421

Quade N, Mendonca C, Herbst K, Heroven AK, Ritter C, Heinz DW, & Dersch P (2012) Structural basis for intrinsic thermosensing by the master virulence regulator RovA of *Yersinia*. *J Biol Chem* **287**: 35796-35803

Raimann E, Schmid B, Stephan R, & Tasara T (2009) The alternative sigma factor σ^L of *L. monocytogenes* promotes growth under diverse environmental stresses. *Foodborne Pathog Dis* **6**: 583-591

Raina S, Missiakas D, & Georgopoulos C (1995) The *rpoE* gene encoding the $\sigma^E(\sigma^{24})$ heat shock sigma factor of *Escherichia coli*. *EMBO J* **14**: 1043-1055

Raivio TL (2005) MicroReview: Envelope stress responses and Gram-negative bacterial pathogenesis. *Mol Microbiol* **56**: 1119-1128

Rakin A, Schneider L, & Podladchikova O (2012) Hunger for iron: the alternative siderophore iron scavenging systems in highly virulent *Yersinia*. *Front Cell Infect Microbiol* **2**

Reitzer L (2003) Nitrogen assimilation and global regulation in *Escherichia coli*. *Annu Rev Microbiol* **57**: 155-176

Resch A, Vecerek B, Palavra K, & Blasi U (2010) Requirement of the CsdA DEAD-box helicase for low temperature riboregulation of *rpoS* mRNA. *RNA Biol* **7**: 796-802

Revell PA & Miller VL (2000) A chromosomally encoded regulator is required for expression of the *Yersinia enterocolitica inv* gene and for virulence. *Mol Microbiol* **35**: 677-685

Rhodius VA, Suh WC, Nonaka G, West J, & Gross CA (2006) Conserved and variable functions of the σ^E stress response in related genomes. *PLoS Biol* **4**: e2

Rimhanen-Finne R, Niskanen T, Hallanvuo S, Makary P, Haukka K, Pajunen S, Siitonen A, Ristolainen R, Pöyry H, Ollgren J, & Kuusi M (2009) *Yersinia pseudotuberculosis* causing a large outbreak associated with carrots in Finland, 2006. *Epidemiol Infect* **137**: 342-347

Riot B, Berche P, & Simonet M (1997) Urease is not involved in the virulence of *Yersinia pseudotuberculosis* in mice. *Infect Immun* **65**: 1985-1990

Rosenzweig JA, Weltman G, Plano GV, & Schesser K (2005) Modulation of *Yersinia* type three secretion system by the S1 domain of polynucleotide phosphorylase. *J Biol Chem* **280**: 156-163

Rosqvist R, Hakansson S, Forsberg A, & Wolf-Watz H (1995) Functional conservation of the secretion and translocation machinery for virulence proteins of yersiniae, salmonellae and shigellae. *EMBO J* **14**: 4187-4195

Rosqvist R, Magnusson KE, & Wolf-Watz H (1994) Target cell contact triggers expression and polarized transfer of *Yersinia* YopE cytotoxin into mammalian cells. *EMBO J* **13**: 964-972

Rozen S & Skaletsky H (2000) Primer3 on the WWW for general users and for biologist programmers. In *Bioinformatics Methods and Protocols: Methods in Molecular Biology,* Krawetz S & Misener S (eds) pp 365-386. Humana Press: Totowa, NJ

Saisawa K (1913) Über die Pseudotuberkulose beim Menschen. *Z Hyg Infektionskr* **73**: 353-400

Savin C, Martin L, Bouchier C, Filali S, Chenau J, Zhou Z, Becher F, Fukushima H, Thomson NR, & Scholz HC (2014) The *Yersinia pseudotuberculosis* complex: Characterization and delineation of a new species, *Yersinia wautersii*. *Int J Med Microbiol* **304**: 452-463

Schiemann D (1979) Synthesis of a selective agar medium for *Yersinia enterocolitica*. *Can J Microbiol* **25**: 1298-1304

Schmiel DH, Young GM, & Miller VL (2000) The *Yersinia enterocolitica* phospholipase gene *yplA* is part of the flagellar regulon. *J Bacteriol* **182**: 2314-2320

Shiozawa K, Nishina T, Miwa Y, Mori T, Akahane S, & Ito K (1991) Colonization in the tonsils of swine by *Yersinia enterocolitica*. *Contrib Microbiol Immunol* **12**: 63-67

Skurnik M, Rådström P, Knutsson R, Segerman B, Hallanvuo S, Thisted Lambertz S, Korkeala H, and Fredriksson-Ahomaa M (2009) *Yersinia*. In *Molecular detection of foodborne pathogens,* Liu D (ed) pp 501-518. CRC Press: Boca Raton, Florida

Sledjeski DD, Gupta A, & Gottesman S (1996) The small RNA, DsrA, is essential for the low temperature expression of RpoS during exponential growth in *Escherichia coli*. *EMBO J* **15**: 3993-4000

Slee KJ & Skilbeck NW (1992) Epidemiology of *Yersinia pseudotuberculosis* and *Y. enterocolitica* infections in sheep in Australia. *J Clin Microbiol* **30**: 712-715

Slee K, Brightling P, & Seiler R (1988) Enteritis in cattle due to *Yersinia pseudotuberculosis* infection. *Aust Vet J* **65**: 271-275

Soutourina OA & Bertin PN (2003) Regulation cascade of flagellar expression in Gram-negative bacteria. *FEMS Microbiol Rev* **27**: 505-523

Sprague LD, Scholz HC, Amann S, Busse HJ, & Neubauer H (2008) *Yersinia similis* sp. nov. *Int J Syst Evol Microbiol* **58**: 952-958

Sprague LD & Neubauer H (2005) *Yersinia aleksiciae* sp. nov. *Int J Syst Evol Microbiol* **55**: 831-835

Staden R (1996) The Staden sequence analysis package. *Mol Biotechnol* **5**: 233-241

Stock AM, Robinson VL, & Goudreau PN (2000) Two-component signal transduction. *Annu Rev Biochem* **69**: 183-215

Storz G & Imlay JA (1999) Oxidative stress. *Curr Opin Microbiol* **2**: 188-194

Sun YC, Koumoutsi A, & Darby C (2009) The response regulator PhoP negatively regulates *Yersinia pseudotuberculosis* and *Yersinia pestis* biofilms. *FEMS Microbiol Lett* **290**: 85-90

Suutari M & Laakso S (1994) Microbial fatty acids and thermal adaptation. *Crit Rev Microbiol* **20**: 285-328

Tamura K, Dudley J, Nei M, & Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**: 1596-1599

Tauxe RV (2004) Salad and *pseudoappendicitis*: *Yersinia pseudotuberculosis* as a foodborne pathogen. *J Infect Dis* **189**: 761-763

Tertti R, Granfors K, Lehtonen OP, Mertsola J, Makela AL, Valimaki I, Hanninen P, & Toivanen A (1984) An outbreak of *Yersinia pseudotuberculosis* infection. *J Infect Dis* **149**: 245-250

Thoerner P, Kingombe CIB, Bögli-Stuber K, Bissig-Choisat B, Wassenaar T, Frey J, & Jemmi T (2003) PCR detection of virulence genes in *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* and investigation of virulence gene distribution. *Appl Environ Microbiol* **69**: 1810-1816

Thomson NR, Howard S, Wren BW, Holden MT, Crossman L, Challis GL, Churcher C, Mungall K, Brooks K, & Chillingworth T (2006) The complete genome sequence and comparative genome analysis of the high pathogenicity *Yersinia enterocolitica* strain 8081. *PLoS Genet* **2**: e206

Tsang TM, Wiese JS, Felek S, Kronshage M, & Krukonis ES (2013) Ail proteins of *Yersinia pestis* and *Y. pseudotuberculosis* have different cell binding and invasion activities. *PLoS ONE* **8**: e83621

Tsubokura M, Otsuki K, Sato K, Tanaka M, Hongo T, Fukushima H, Maruyama T, & Inoue M (1989) Special features of distribution of *Yersinia pseudotuberculosis* in Japan. *J Clin Microbiol* **27**: 790-791

Turner AMW, Love CF, Alexander RW, & Jones PG (2007) Mutational analysis of the *Escherichia coli* DEAD box protein CsdA. *J Bacteriol* **189**: 2769-2776

Uchiyama T, Miyoshi-Akiyama T, Kato H, Fujimaki W, Imanishi K, & Yan XJ (1993) Superantigenic properties of a novel mitogenic substance produced by *Yersinia pseudotuberculosis* isolated from patients manifesting acute and systemic symptoms. *J Immunol* **151**: 4407-4413

Uppal S, Rao Akkipeddi VSN, & Jawali N (2008) Posttranscriptional regulation of *cspE* in *Escherichia coli*: involvement of the short 5'-untranslated region. *FEMS Microbiol Lett* **279**: 83-91

Wachtel MR & Miller VL (1995) *In vitro* and *in vivo* characterization of an *ail* mutant of *Yersinia enterocolitica*. *Infect Immun* **63**: 2541-2548

Walker SJ, Archer P, & Banks JG (1990) Growth of *Yersinia enterocolitica* at chill temperatures in milk and other media. *Milchwissenschaft* **45**: 503-506

Wauters G (1973) Improved methods for the isolation and the recognition of *Yersinia enterocolitica*. *Contrib Microbiol Immunol* **2**: 68-70

Weagant SD & Feng P (2001) *Yersinia enterocolitica*. In *Bacteriological Analytical Manual,* Hammack T, Feng P, Jinneman K, Regan PM, Kase J, Orlandi P, Burkhardt W (eds) U.S. Food and Drug Administration

Weber H, Polen T, Heuveling J, Wendisch VF, & Hengge R (2005) Genome-wide analysis of the general stress response network in *Escherichia coli*: σ^S-dependent genes, promoters, and sigma factor selectivity. *J Bacteriol* **187**: 1591-1603

West AH & Stock AM (2001) Histidine kinases and response regulator proteins in two-component signaling systems. *Trends Biochem Sci* **26**: 369-376

White-Ziegler CA, Um S, Perez NM, Berns AL, Malhowski AJ, & Young S (2008) Low temperature (23°C) increases expression of biofilm-, cold-shock-and RpoS-dependent genes in *Escherichia coli* K-12. *Microbiology* **154**: 148-166

Wösten M (1998) Eubacterial sigma-factors. *FEMS Microbiol Rev* **22**: 127-150

Wren BW (2003) The yersiniae—a model genus to study the rapid evolution of bacterial pathogens. *Nat Rev Microbiol* **1**: 55-64

Wren BW & Tabaqchali S (1990) Detection of pathogenic *Yersinia enterocolitica* by the polymerase chain reaction. *Lancet* **336**: 693

Xia B, Ke H, & Inouye M (2001) Acquirement of cold sensitivity by quadruple deletion of the *cspA* family and its suppression by PNPase S 1 domain in *Escherichia coli*. *Mol Microbiol* **40**: 179-188

Xia B, Ke H, Jiang W, & Inouye M (2002) The cold box stem-loop proximal to the 5'-end of the *Escherichia coli cspA* gene stabilizes its mRNA at low temperature. *J Biol Chem* **277**: 6005-6011

Xia B, Ke H, Shinde U, & Inouye M (2003) The role of RbfA in 16S rRNA processing and cell growth at low temperature in *Escherichia coli*. *J Mol Biol* **332**: 575-584

Yamamoto K, Hirao K, Oshima T, Aiba H, Utsumi R, & Ishihama A (2005) Functional characterization *in vitro* of all two-component signal transduction systems from *Escherichia coli*. *J Biol Chem* **280**: 1448-1456

Yamanaka K & Inouye M (2001) Selective mRNA degradation by polynucleotide phosphorylase in cold shock adaptation in *Escherichia coli*. *J Bacteriol* **183**: 2808-2816

Yamanaka K, Mitta M, & Inouye M (1999) Mutation analysis of the 5'untranslated region of the cold shock *cspA* mRNA of *Escherichia coli*. *J Bacteriol* **181**: 6284

Yamanaka K & Inouye M (2001) Induction of CspA, an *E. coli* major cold-shock protein, upon nutritional upshift at 37°C. *Genes Cells* **6**: 279-290

Yang Y & Isberg RR (1993) Cellular internalization in the absence of invasin expression is promoted by the *Yersinia pseudotuberculosis yadA* product. *Infect Immun* **61**: 3907-3913

Yang Y, Merriam JJ, Mueller JP, & Isberg RR (1996) The *psa* locus is responsible for thermoinducible binding of *Yersinia pseudotuberculosis* to cultured cells. *Infect Immun* **64**: 2483-2489

Young GM & Miller VL (1997) Identification of novel chromosomal loci affecting *Yersinia enterocolitica* pathogenesis. *Mol Microbiol* **25**: 319-328

Zhang W, Wang Y, Song Y, Wang T, Xu S, Peng Z, Lin X, Zhang L, & Shen X (2013) A type VI secretion system regulated by OmpR in *Yersinia pseudotuberculosis* functions to maintain intracellular pH homeostasis. *Environ Microbiol* **15**: 557-569

Zheng XB, Tsubokura M, Wang Y, Xie C, Nagano T, Someya K, Kiyohara T, Suzuki K, & Sanekata T (1995) *Yersinia pseudotuberculosis* in China. *Microbiol Immunol* **39**: 821-824