Aus dem Institut für
Lebensmittelhygiene des
Fachbereichs Veterinärmedizin der
Freien Universität Berlin

Prevalence of *Yersinia enterocolitica* in retail seafood and the isolate specific cold response in transcriptional, proteomic, and membrane physiological changes

Inaugural-Dissertation
zur Erlangung des Grades eines
PhD of Biomedical Sciences
an der
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vorgelegt von
Chenyang Li
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LIST OF ABBREVIATIONS

2-DE	2-dimensional polyacrylamide gel electrophoresis
BfR	Federal Institute for Risk Assessment
CDC	Centers for Disease Control and Prevention
CFU	Colony forming unit
Cip	Cold induced proteins
CSC box	Cold shock cut box
CSD	Cold shock domain
Csp	Cold shock protein
EEA	European economic area
FDA	Food and Drug Administration
FoodNet	The foodborne diseases active surveillance network
ISO	International Standard Organization
iTRAQ	Isobaric tag for relative and absolute quantitation
LB	Luria-bertani
MALDI-ToF/MS	Matrix-assisted laser desorption ionization time-of-flight mass spectrometry
MLVA	Multilocus variable-number tandem-repeat analysis
mRNA	Messenger RNA
MS/MS	Mass fingerprinting and tandem mass spectrometry
NA	Not available
O-ag	O-antigen
OMPs	Outer membrane proteins
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PTS	Phosphotransferase system
qPCR	Real-time quantitative polymerase chain reaction
RIN	Rna integrity number
rRNA	Ribosomal-RNA
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
TCA	Tricarboxylic acid
tRNA	Transfer-RNA
WTO	World trade organization

CHAPTER 1 ABSTRACT FOR THE WHOLE RESEARCH

Yersinia (*Y.*) *enterocolitica* is a zoonotic enteropathogenic bacterium which can cause acute gastroenteritis and mesenteric inflammation. The life cycle of *Y. enterocolitica* comprises multiplication in mammalian hosts and survival outside the host. In natural environments, it is commonly found in animals, food and the aquatic and terrestrial environment. However, the reservoirs and transmission routes of this pathogen are still poorly understood. High prevalences have been reported in a variety of food products, but limited data is available for prevalences in retail food products, e.g. seafood, vegetables and other fresh products. The aim of this study was to determine the prevalence of *Y. enterocolitica* in seafood. Seafood samples were purchased randomly from retail shops in Berlin (Germany). The presence of *Y. enterocolitica* was examined with cold enrichment followed by cultivation on selective agar. Presumptive *Y. enterocolitica* isolates were analyzed by biotyping, serotyping and antimicrobial susceptibility testing. The total prevalence of *Y. enterocolitica* in seafood samples was 2.7% (6/220): mussels (2/90), shrimp (1/89) and cephalopod (3/41). All isolates belonged to biotype 1A and three isolates were identified as serotype O:8, one isolate as O:5,27, while two samples did not belong to the investigated serotypes. And most isolates (4/6) contained the virulence-associated genes *inv* and *ystB*. *Y. enterocolitica* isolates were susceptible to cefotaxime, cefuroxime, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, tetracycline and trimethoprim. Resistance was observed for cephalothin (83.3%), amoxicillin (83.3%) and ampicillin (50.0%). This study provides the first comprehensive analysis of *Y. enterocolitica* in retail seafood in Germany. Although the determined prevalence was not high, and all isolates belonged to biotype 1A, the study indicated that seafood might be a potential source of infection by *Y. enterocolitica*.

Refrigeration temperature is widely used for food storage and production, especially on retail level. As an important foodborne pathogen, *Y. enterocolitica* is able to survive at low temperature. So that its psychrotrophic ability increases the risk of infection through contaminated food. Although the general cold response profiles are well described, the mechanisms of cold adaptation have not been fully investigated. In this study, isolates were collected from various matrices including humans, animals and food, covering different biogroups and serogroups. Growth profiles were tested and compared at 4 °C for selected isolates. Cold-response genes which differed in function were selected to study the association between cold response and gene expression. Global proteomic analysis was investigated with label-free quantification. In addition, the bacterial motility and membrane fluidity were investigated to demonstrate the relationship between membrane physiological changes and cold response. According to the results, strain specific cold response characteristics were detected. The expressional changes of cold-response genes showed that the ability to survive in response to cold demands the capacity to adapt to cold. This cold adaptation on transcriptional level is generated by the repression of cold-shock genes and induction of the other cold-acclimation genes during prolonged growth. Proteomic analysis identified cold-response proteins, in which the predominant functional categories are associated with protein synthesis, cell membrane parts and cell motility. Additionally, the physiological processes in cell fluidity and motility might be responsible for growth at low temperatures. Summarized, by combining different approaches, cold response was described systematically, providing a better understanding of the physiological processes of *Y. enterocolitica* in response to cold stress.

CHAPTER 2 INTRODUCTION

2.1 Taxonomy, history and morphology of *Yersinia*

Yersinia is a bacterial genus that constitutes of at least 18 recognized species: *Y. pestis*, *Y. pseudotuberculosis*, *Y. enterocolitica*, *Y. frederiksenii*, *Y. intermedia*, *Y. kristensenii*, *Y. bercovieri*, *Y. mollaretii*, *Y. rohdei*, *Y. aldovae*, *Y. ruckeri*. *Y. aleksiciae*, *Y. bercovieri*, *Y. entomophaga*, *Y. massiliensis*, *Y. pekkanenii*, *Y. philomiragia* and *Y. similis* [1-12].

Y. pestis, as the first known species, was identified in 1894 by Alexandre Yersin, a Swiss bacteriologist [13]. In 1944, van Loghem reclassified the species *P. pestis* and *P. rondentium* into a new genus, *Yersinia* [14]. Following the introduction of the bacteriological code, it was accepted as valid in 1980 [15]. The first recognized reference to *Y. enterocolitica* was made in the United States in 1934 by McIver and Pike [16]. In 1964, Frederiksen set the taxonomic stage of *Y. enterocolitica* by introducing it within the family *Enterobacteriaceae* [17].

Yersinia species are Gram-negative, facultative anaerobic and rod-shaped non-spore forming bacteria with a width of 0.5 to 0.8 μm and a length of 1.0 to 3.0 μm. They are oxidase-negative and can ferment glucose and reduce nitrates to nitrites [18]. *Yersinia* normally produces peritrich arranged flagella, which is probably synthesized in the same way as other peritrichously flagellated bacteria and has the ability to move [19-23].

2.2 Clinical relevance

2.2.1 Pathogenicity

Some members of the genus *Yersinia* are well-recognized as human and animal pathogens. Among them, only *Y. pestis*, *Y. pseudotuberculosis* and certain strains of *Y. enterocolitica* show pathogenicity and they are highly important as pathogens for humans and animals. The species and their pathogenicity are listed in **Table 1**.

Table 1: *Yersinia* species and pathogenicity [24-26]

Species	Pathogenicity
Y. enterocolitica *Y. pseudotuberculosis*	Enteric Yersiniosis
Y. pestis	Plague
Y. ruckeri	Fish pathogen
Y. aldovae, Y. aleksiciae, Y. bercovieri, Y. entomophaga, Y. frederiksenii, Y. intermedia, Y. kristensenii, Y. massiliensis, Y. mollaretii, Y. nurmii, Y. pekkanenii, Y. philomiragia, Y. rohdei, Y. similis	Non-pathogenic

The plague caused by *Y. pestis*, is recognized as one of the most devastating bacterial diseases in the history of mankind. The bacterium was responsible for millions of human mortalities during multiple pandemics [27]. Yersiniosis caused by *Y. enterocolitica* and (to a lesser degree) *Y. pseudotuberculosis* is a typical self-limiting gastrointestinal disease, affecting human and animal populations.

In the European Union, *Y. enterocolitica* is the third most common enteric bacterial pathogen after *Campylobacter* and *Salmonella* [28, 29]. And it is the fifth most common enteric bacterial cause of foodborne illness in the United States [30]. The infections of human with *Y. enterocolitica* are mainly caused by ingestion of contaminated food, e.g. by insufficiently heated or raw consumed pork and milk [31-33] or contaminated drinking water. Rarely, the infection spreads between humans [34].

The infection of *Y. enterocolitica* is usually characterized by a self-limiting acute infection at the beginning in the intestine and spreading to the mesenteric lymph nodes. However, more serious infections and chronic conditions can also occur, particularly in immunocompromised individuals [35]. According to the FDA, the medium infective dose of *Y. enterocollitica* for humans is generally estimated in the range of 10^4 – 10^6 microorganisms [36]. The clinical presentation of symptoms may depend on bacterial strain specifics and host factors.

2.2.2 Detection, transmission and medical treatment

Yersiniosis as a noteworthy disease mainly caused by *Y. enterocolitica*, can affect all humans, especially the children under the age of 5 years, the elderly and people with reduced immunity. However, the infection of *Y. enterocolitica* is often resembling with nonspecific symptoms making many cases of Yersiniosis misdiagnosed [23].

Several phenotypic and genotypic methods have been developed to reliably detect *Y. enterocolitica*. However, cases of Yersiniosis were sometimes under-estimated due to the difficulty in detection [37-39]. Moreover, since *Y. enterocolitica* shares similarities with other enteric bacteria, it is difficult to detect this pathogen especially in food samples. Therefore, the methods suitable for in-situ detection and characterization developed. The current methods for detection of *Y. enterocolitica* are cultural methods, numerous

immunological and molecular techniques [40]. Based on the standardized reference methods designated by the International Standard Organization (ISO10273:2003), method for detection of presumptive pathogenic *Y. enterocolitica* in foods includes parallel use of the enrichment in PSB broth and secondary enrichment in ITC broth and plating on CIN or SSDC [41]. Meanwhile, the limitations of culture-based methods existed as they are time-consuming, tedious, and do not provide information to strain subtype and it can't be used for high-throughput detection. So that the immunological and molecular methods have been adapted, providing rapid and more sensitive methods. For example, immunomagnetic separation (IMS) is used for the identification of pathogens and it eliminates the enrichment and reduces the detection time. Enzyme-linked immunosorbent assay (ELISA) has also been developed for the detection of *Y. enterocolitica* from clinical, environmental, and food samples [42]. Lately, microarray has emerged as a sensitive and effective way for the detection of *Y. enterocolitica* in clinical, environmental and food samples [43] as a highly specific and sensitive method is most commonly used as the nucleic DNA-based technique for the diagnosis of foodborne pathogens [44]. MALDI-TOF-MS has been reported as a reliable technique for the rapid identification of *Y. enterocolitica* strains. It allows the subtyping of strains to the biotype level with high speed and easy applicability [45]. Molecular subtyping has also been studies in *Y. enterocolitica* with a large number of subgroups according to biochemical characterization. Biotyping associated with pathogenicity and serotyping based on lipopolysaccharide surface O antigen have been described to determine the phenotypic characteristics [46]. Several techniques are available for *Y. enterocolitica* typing according to amplification-based and sequencing-based methods. ERIC-PCR has been applied for screening potential virulence, pathogenicity and genotyping of *Y. enterocolitica* strains according to virulence markers and geographical origin [47, 48]. Multilocus sequence typing (MLST) is applied by examining genetic relatedness among strains. The nucleotide sequences of multiple housekeeping genes were analyzed within a species and the resulted sequence types (STs) are compared for nucleotide substitution. MLST is effective in distinguishing isolates with a more recent genetic divergence [49]. In addition, Whole genome sequencing (WGS) also used to determine the relatedness of bacteria by performing interspecies genomic comparison between species [50]. Benefits of WGS include the possibility to perform highly discriminatory analyses and retrieving results for various genetic analyses. Therefore, numerous methods are available for epidemiological surveillance and phylogenetic studies of *Y. enterocolitica* meanwhile, the selection of the most appropriate typing approach should depends on the purpose.

Infection by *Y. enterocolitica* often caused by eating contaminated food, such as contaminated vegetables and salad, raw pork intestines (chitterlings) and unpasteurized milk or untreated water. Occasionally, *Y. enterocolitica* infection occurs after contact with infected animals. On rare occasions, it can be transmitted as a result of the bacterium passing from the stools or soiled fingers of one person to the mouth of another person. This may happen when basic hygiene and hand washing habits are inadequate [23, 51].

Normally, the majority of the gastrointestinal infections are self-limiting and antimicrobial therapy is usually not recommended of *Y. enterocolitica* in an immunocompetent host. However, immunocompromised patients need special attention and antibiotic treatment since the mortality of them can be as high as 50% in the hosts and patients with septicemia or invasive infection. Hence, special attention and antimicrobial therapies are warranted. According to WHO recommendations, the antibiotic therapy of *Y. enterocolitica* is tetracycline, chloramphenicol, gentamycin and cortimoxazole [149].

Recently, the third generation cephalosporin and fluoroquinolones, which have excellent *in vitro* activity, have been considered as better alternatives [52]. They have led to a significant decrease of mortality due to septicemia and the use of fluoroquinolones particularly is associated with a higher cure rate of fever.

Y. enterocolitica was reported to be highly susceptible to most antibiotics except penicillin, ampicillin, amoxicillin-clavulanic acid and the first-generation cephalosporins [53, 54]. However, the prevalence of drug-resistant *Y. enterocolitica* strains has increased in recent years, due to the overuse of antibiotics and bacteria or genes transmission among different species [55]. For example, resistance to aminoglycosides (gentamicin and tobramycin), fluoroquinolones (ciprofloxacin) and intermediate resistance to the third generation cephalosporins (cefatriaxone) was illustrated in in retail poultry meat and swine feces in China [56]. According to previous study, the third generation cephalosporins used to be the best therapeutic options warranted to treat enterocolitis in compromised hosts and in patients [57]. However, due to the novel drug-resistance phenotype arising in *Y. enterocolitica*, it might lead to clinical failures when administering inappropriate antibiotic to treat *Yersinia* infections. In addition, increasing trend of streptomycin resistance was also reported in retail foods in China and pigs at slaughter in Italy [55, 58]. So that the problem on antimicrobial resistance of *Y. enterocolitica* has become more serious.

2.3 Epidemiology of *Yersinia enterocolitica*

2.3.1 Prevalence in animal

Y. enterocolitica has been detected from diverse animal sources: farm animals, domestic pets and free-living livestock [28, 59]. In addition, in wild animals, *Y. enterocolitica* has also been isolated from the intestinal tract of many species especially wild boars [60, 61]. Various studies in Europe showed the prevalence of *Y. enterocolitica* (17% to 35%) in wild boars [60, 62, 63]. In addition, the prevalence of other animals has been investigated. *Y. enterocolitica* in deer in Poland are 21.6% in red deer (63/291), 9.4% in roe deer (11/117) and 13.3% in fallow deer (2/15) [64]. In the year of 2003, Niskanen et al. (2003) found 26 (5.6%) wild birds harboring *Y. enterocolitica* in 468 examined Swedish birds [65]. It was found in the alpine accentor, one of the bird species in high mountain areas, 73% of the detected samples were positive for *Y. enterocolitica* in the Tatra Mountains in 2007 [66]. *Y. enterocolitica* was isolated from 15.7% (88/560) of wild rodents in Japan [67].

According to the report of the German Federal Institute for Risk Assessment, the percentages of *Y. enterocolitica* positive records in animals between 2011 and 2016 are presented in Germany in **Table 2**. The main reservoir were bovines, pigs and goats. And it showed that very few *Y. enterocolitica* were detected in horses, cats or mice. Between 2011 and 2016, there is mainly a decrease in the prevalence. However in the year of 2014 and 2015, high percentage rates were detected in some animals, especially the prevalence in laying hens (18.18% in flock group and 13.33% in the individual animal group) in 2014 and the rates of the goats in herd group (15.38%) in 2015.

Table 2: Prevalence of *Y. enterocolitica* in animals (2011 – 2016)

Animals	2011 [68]	2012 [69]	2013 [70]	2014 [71]	2015[72]	2016[73]
Herd						
Laying Hens	0% (0/65)	0% (0/56)	NA	18.18% (4/22)	0% (0/10)	0% (0/10)
Broilers	NA	0% (0/32)	NA	0% (0/18)	NA	0% (0/2)
Bovines	14.63% (30/205)	1.91% (4/209)	0.69% (6/625)	4.27% (7/164)	1.86% (3/161)	1.14% (2/175)
Pigs	12.17%(14/125)	2.73% (3/110)	0.58% (1/171)	1.60% (2/125)	5.7% (4/70)	1.12% (1/89)
Sheep	5.77% (3/52)	0% (0/50)	0% (0/50)	4.26% (2/47)	0% (0/30)	4.69% (3/64)
Goats	14.29% (2/14)	18.18% (2/11)	0% (0/20)	6.67% (1/15)	15.38% (2/13)	0% (0/24)
Horses	0% (0/32)	0% (0/24)	0% (0/44)	0% (0/17)	0% (0/11)	2.94%(1/34)
Individual animals						
Chicken	0% (0/1042)	0% (0/239)	NA	0% (0/1027)	0% (0/1421)	0% (0/1574)
Laying Hens	0% (0/92)	0% (0/89)	0% (0/55)	13.33% (4/30)	0% (0/25)	0% (0/42)
Broilers	NA	0% (0/70)	0% (0/12)	0% (0/58)	NA	0% (0/10)
Turkey	0% (0/25)	0% (0/465)	0% (0/16)	0% (0/206)	0% (0/221)	0% (0/266)
Poultry	5.26% (1/19)	0% (0/114)	0% (0/116)	0% (0/544)	0.35% (1/284)	0% (0/332)
Bovines	1.91% (86/4512)	1.20% (32/2670)	1.33% (24/1811)	3.16% (84/ 2660)	1.60% (33/ 2063)	0.13% (5/ 3964)
Pigs	1.26% (32/2539)	0.29% (6/2101)	4.97%(54/1086)	1.99% (44/2215)	3.83% (61/1593)	0.67% (11/1639)
Sheep	1.29% (4/310)	0% (0/416)	1.21% (3/247)	0.77% (3/389)	0.73% (2/274)	1.29% (5/388)
Goats	3.73% (5/134)	3.00% (3/100)	0% (0/104)	3.57% (4/112)	2.59% (3/116)	1.45% (2/138)
Horses	0% (0/991)	0% (0/1965)	0% (0/116)	0% (0/2067)	0% (0/2146)	0.13% (3/2379)
Dogs	2.28 % (7/307)	0.62% (10/1616)	1.78% (3/169)	0.90% (17/1898)	1.25% (25/2007)	0.95% (22/2315)
Cats	0% (0/247)	0.1% (1/974)	0% (0/109)	0.08% (1/1179)	0.27% (3/1103)	0.08% (1/1287)
Mice	NA	NA	NA	0% (0/413)	NA	NA
Wild animals	0.90% (1/111)	4.00% (3/75)	NA	1.90% (4/210)	4.76% (6/126)	0% (0/155)
Other animals	0.11% (3/2639)	0.26% (7/2740)	0.35% (4/1133)	0.63% (21/3358)	0.51% (13/2549)	1.06% (31/2917)

Meanwhile, in China, six strains were isolated from domestic dogs, which shared the same patterns as strains isolated from diarrhea patients. This indicated that the strains from domestic dogs have a close correlation with the strains causing human infections

[74]. Furthermore, *Y. enterocolitica* has also been isolated from flies in farm piggeries and kitchens [75], suggesting that insects can also act as vector.

2.3.2 Prevalence in environment

Y. enterocolitica is ubiquitous in nature and can be routinely isolated from a broad range of terrestrial and freshwater ecosystems including soil, plants, lakes, rivers, wells and streams [76-80]. In Brazil, *Y. enterocolitica* was found distributed in oceans, waterfalls, polluted rivers, sewage and fresh water [76]. The frequency of the positive isolates collected in the upstream section of the Drwezca River in Poland is around 10.26% [78]. In the U.S., 8.25% of *Y. enterocolitica* (25/303) were detected in different origin of the catchment area of the Lower Chippewa River [81].

In addition, there are dependencies between the season and the samples collection and the quantitative detection of *Y. enterocolitica*. In winter, more colony-forming samples were collected than in summer. This might due to the advantage *Y. enterocolitica* has compared with other microorganisms that can better assert itself in competition in different environments [82].

2.3.3 Prevalence in food

Evidence for the presence of *Y. enterocolitica* in a variety of foods including milk and milk products, raw meat (beef, pork, and lamb), poultry, eggs, vegetables, bean sprouts, tofu and seafood has been provided [83]. Pork is the main food reservoir of *Y. enterocolitica* [84-87]. According to the Federal Institute for Risk Assessment (BfR), high prevalence of *Y. enterocolitica* in pork was reported during the six years from 2011 to 2016 (2011: 8.76 %, 2012: 7.95 %, 2013: 2.11 %, 2014: 1.43%, 2015: 10.48 % and 2016: 30.14%). The prevalence of *Y. enterocolitica* in pork decreased by years during the first four years, but since 2015, the high prevalence in pork appeared and sustained over the following years. With the improvement of detection methods, the increased prevalence might be associated with the detected methods with higher accuracy and sensitivity. Nevertheless, the surveillance and the prevention of *Y. enterocolitica* must not be ignored. **Table 3** shows the prevalence rates of the different pork and pork products during these years.

Table 3: Prevalence of *Y. enterocolitica* in pork and pork products in Germany (2011 – 2016)

Pork product	2011 [68]	2012 [69]	2013 [70]	2014 [71]	2015 [72]	2016 [73]
Pork	8.76% (12/137)	7.95% (12/151)	2.11% (4/190)	1.43% (4/280)	10.48% (11/105)	30.14% (22/73)
Raw pork	1.89% (1/53)	20% (1/5)	NA	9.09% (1/11)	NA	NA
Minced pork	4.6% (4/87)	0% (0/23)	5.17% (9/174)	3.26% (1/21)	4.85% (5/103)	13.45% (16/119)
Processed minced pork	3.36% (10/298)	1.92% (1/52)	0% (0/31)	NA	NA	NA

Additionally, the detection of *Y. enterocolitica* in different food types between 2011 and 2016 are shown in **Table 4**. High prevalence in food in the latest two years 2015 and 2016 occurred, especially the numbers of positive samples in meat (excluding poultry),

minced meat and some processed minced meat in 2015 (19.20%, 11.69% and 23.68%). In 2016, the number of positive samples in processed minced meat was still high, which was consistent with the high prevalence in pork and pork products since 2015.

Table 4: Prevalence of *Y. enterocolitica* in food products in Germany (2011 – 2016)

Examined food	2011[68]	2012[69]	2013[70]	2014[71]	2015[72]	2016[73]
Meat exclude poultry	6.22% (13/209)	7.33% (14/191)	1.6% (4/250)	1.08% (4/370)	19.20% (24/125)	6.63% (12/181)
Raw meat, pieces	50% (1/2)	20% (1/5)	NA	6.25% (1/16)	50% (1/2)	NA
Raw meat, crushed	0% (0/66)	NA	NA	NA	NA	0% (0/10)
Minced meat	2.9% (4/138)	3.45% (1/29)	5.29% (11/208)	3.70% (5/135)	11.69% (18/154)	4.29% (6/140)
Processed minced meat	12.81% (47/367)	6.35% (19/299)	5.34% (15/281)	7.33% (59/805)	5.15% (14/272)	12.13% (89/734)
Heat-treated meat products	8.33% (1/12)	NA	NA	NA	NA	NA
Stabilized meat products	16.67% (1/6)	2.94% (1/34)	1.33% (1/75)	0% (0/72)	1.45% (1/69)	0% (0/115)
Poultry meat, total	NA	NA	0% (0/16)	0% (0/56)	NA	NA
Fish, fish Product & seafood	NA	NA	NA	0% (0/12)	NA	NA
Certified milk	0% (0/33)	0% (0/40)	30.77% (4/13)	0% (0/75)	0% (0/27)	0% (0/26)
Raw milk from the farm	NA	7.14% (1/14)	0% (0/19)	9.09% (1/11)	0% (0/12)	7.14% (1/14)
Collected milk (raw milk)	7.69% (2/26)	8.33% (1/12)	0% (0/11)	20% (1/5)	NA	3.45% (1/29)
Raw milk of other species	0% (0/30)	0% (0/23)	0% (0/18)	2.78% (1/36)	0% (0/26)	13.64% (3/22)
Fresh fruits	NA	NA	0% (0/59)	NA	NA	NA
Delicatessen salad	NA	50% (1/2)	NA	NA	0% (0/36)	NA
Infant food up to 6 months	NA	0% (0/15)	NA	NA	0% (0/14)	NA
Leafy vegetables	NA	0.87% (1/115)	NA	NA	NA	NA
Fresh vegetables	NA	0% (0/38)	NA	0% (0/15)	0% (0/20)	0% (0/84)
Other food	NA	NA	NA	8.33% (1/12)	NA	NA

In addition, during the slaughter and processing of meat, *Y. enterocolitica* may also be transferred from contaminated tissues onto other meat parts. The bacteria are notably contained in the tonsils, the lymph nodes and the intestine, while the meat from the areas close to the head and sternum is the most exposed [88, 89]. Hence, the

contamination from the infected parts to body intended for consumption should be avoided during the slaughtering process. In addition, the surface and equipment properly have the risk of cross-contamination with processing food product [23]. Due to the extensive spread of this bacterium, the prevention and surveillance should be built.

2.3.4 Prevalence in human

Various outbreaks of *Y. enterocolitica* are displayed in the past several years worldwide. These outbreaks were largely caused by consumption of contaminated food products. In **Table 5**, the main outbreaks were listed in the last few years since 1990.

Table 5： Main outbreaks of *Y. enterocolitica* (1990-2019)

Year	Place	Serotype	Number of cases	Infection roots
1990	England	O:10K,O:6,30	36	Pasteurized milk [90, 91]
1995	Vermont and New Hampshire, USA	O:8	10	Pasteurized milk [92]
1997	Tamil Nadu India	O:3	25	Buttermilk [93]
1998	New York, USA	unknown	unknown	Cold cuts [94]
1999	Texas, USA	unknown	unknown	Pork [94]
2000-2001	Georgia, USA	unknown	unknown	Pork [94]
2002	New Mexico, USA	unknown	unknown	Ham salad [94]
2003-2004	Wisconsin and Georgia, USA	unknown	unknown	Pig stomach [81]
2005	Japan	O:8	42	Mixed salad [95]
2005-2006	Norway	O:9/ O:3	15	Brawn [96]
2011	Norway	O:9	21	Packaged salad [97]
2012	Japan	O:8	39	Unknown[98]
2013	Japan	O:8	52	Vegetable salad [98]
2014	Norway	O:9	133	Radicchio Salad [99]
2017-2018	Finland	O:3	55	Unknown [100]
2019	Sweden and Denmark	O:3	57	Fresh spinach [101]

There are several national networks that monitor Yersiniosis reporting the human cases, particularly in the EU and the US. Differences in reporting and isolation methods and availability of strain information greatly complicate comparison of results between different regions [35, 102]. According to the European Centre for Disease Prevention and Control, in the year from 2014 to 2018, confirmed cases of Yersiniosis were reported by EU/EEA countries in **Table 6**. During these years, Germany accounted for the highest number of cases, followed by France.

Table 6: Distribution of confirmed Yersiniosis cases and rates per 100000 populations in EU/EEA (2014–2018) [100]

Country	2014		2015		2016		2017		2018	
	N	Rate	N	Rate	N	Rate	N	Rate	N	Rate
Austria	107	1.3	118	1.4	86	1	95	1.1	136	1.5
Belgium	309	NA	350	3.1	355	3.1	317	2.8	392	3.4
Bulgaria	20	0.3	12	0.2	10	0.1	17	0.2	9	0.1
Croatia	20	0.5	16	0.4	22	0.5	29	0.7	20	0.5
Cyprus	0	0	0	0	0	0	0	0	0	0
Czech Republic	557	5.3	678	6.4	608	5.8	611	5.8	622	5.9
Denmark	250	4.4	273	4.8	278	4.9	206	3.6	282	4.9
Estonia	62	4.7	53	4	45	3.4	43	3.3	63	4.8
Finland	579	10.6	582	10.6	407	7.4	423	7.7	529	9.6
France	574	NA	624	NA	735	NA	738	NA	929	NA
Germany	2470	3.1	2741	3.4	2763	3.4	2580	3.1	1877	2.3
Hungary	43	0.4	41	0.4	70	0.7	30	0.3	36	0.4
Iceland	3	0.9	1	0.3	1	0.3	0	0	2	0.6
Ireland	5	0.1	13	0.3	3	0.1	6	0.1	8	0.2
Italy	18	NA	7	NA	9	NA	8	NA	14	NA
Latvia	28	1.4	64	3.2	47	2.4	47	2.4	68	3.5
Lithuania	197	6.7	165	5.6	155	5.4	174	6.1	139	4.9
Luxem-bourg	19	3.5	15	2.7	12	2.1	15	2.5	16	2.7
Norway	211	4.1	76	1.5	57	1.1	67	1.3	105	2
Poland	212	0.6	172	0.5	167	0.4	191	0.5	170	0.4
Romania	32	0.2	25	0.1	40	0.2	36	0.2	22	0.1
Slovakia	172	3.2	224	4.1	200	3.7	242	4.5	259	4.8
Slovenia	19	0.9	10	0.5	31	1.5	18	0.9	32	1.5
Spain	436	NA	432	NA	514	NA	585	NA	549	NA
Sweden	248	2.6	245	2.5	230	2.3	236	2.4	278	2.7
United Kingdom	58	0.1	44	0.1	87	0.1	142	0.2	198	0.3
EU/EEA	6649	1.8	7005	1.9	6946	1.8	6891	1.8	6806	1.6

Although, incidences have declined over the last 5 years in Germany, there is still some evidence to support the idea that high meat consumption, particularly pork resulted in the high infection in Germany compared to other European nations. According to the latest surveillance report from FoodNet in 2015, the percentage of incidence rate of *Yersinia* in 2015 decreased (59%, 18% and 10%) compared with average annual incidence rate of year 1996 – 1998, 2006 – 2008 and 2011 – 2014 respectively [103]. While, during 2018 in the US, based on the identification of FoodNet, the number of cases was 465 in *Yersinia* and the incidence of infection (per 100,000 population) was 0.9. Compared with the years during 2015 to 2017, the incidence significantly increased (58%) [104]. The prevalence surveys of pathogenic *Y. enterocolitica* were also displayed in 10 regions of China in 7,304 patients <5 years of age with diarrhea. The average

prevalence of *Y. enterocolitica* was 0.59% (43/7,304) and the prevalence was at different levels in different province. And the prevalence calculated for southern China (0.80%) was slightly higher than that for northern China (0.53%) [105].

Furthermore, the infection of *Y. enterocolitca* depends on the age and gender as well. In China, more infections occurred among children from the age of 0.5 to 2 years and more often in boys than in girls (1.63:1) [105]. The age and gender dependent infections have also been reported in the US. Among the total 139 incidence of *Y. enterocolitca* in 2015, the number of the incidence in the children younger than 5 years old was 22 and the incidence rate was 0.74, which has the highest incidence rate among all age groups [103]. Moreover, the number and the rate of incidence in female (87, 0.35) was higher than that in male (52, 0.22) in all the infection [103]. Similarly, the distribution of the Yersiniosis cases related to the age and gender was also investigated in EU/EEA. Among the 6 404 confirmed Yersiniosis cases in 2018, the male-to-female ratio was 1.1:1. The highest notification rate per 100 000 population was detected in the age group 0–4-years. This age group accounted for 23% of the total cases. In **Fig 1**, the distribution of confirmed Yersiniosis cases per 100 000 was presented.

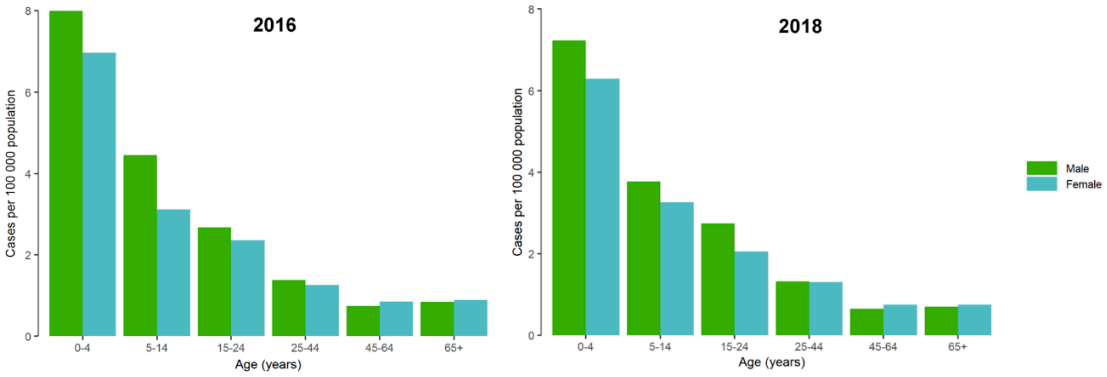

Fig 1: Distribution of confirmed Yersiniosis cases, by age and gender in EU/EEA (2016 and 2018) [100, 106]

According to **Fig 1**, young people are more likely to have Yersiniosis especially under the age of 4. Under the age of 44, more infections occurred in the male people while older than 44, more female infected to this disease. The systematic reports in EU and China showed that, in general, female are more likely to get infection especially in the younger age. However, in the US, more female are likely to get infection to Yersiniosis during investigated years. Hence, the differences in immunological response might explain the different susceptibility to Yersiniosis.

2.4 *Yersinia enterocolitica* characterization

2.4.1 Serotype

Y. enterocolitica has various groups of heterogeneous strains, which are traditionally classified on the basis of phenotypic characteristics. It has more than 57 O serogroups [61, 107-109]. Serotypes O:3, O:9, O:8 and O:5,27 are commonly associated with human Yersiniosis [28, 110]. Among these, serotypes O:3, O:5,27 and O:9 usually produce self-limiting gastroenteritis, whereas O:8 strains are more likely to cause systemic infections [28, 111, 112]. Most of the strains isolated from animal sources differ

serologically from strains isolated from humans [32]. Previous works indicated that pigs and pork products may be the major reservoirs of serotype O:3 [113-116]. Clinical *Y. enterocolitica* isolates from humans predominantly belong to the serotypes O:3 ("European strain"), O:8 ("American strain"), O:9, and O:5,27, with a certain variability among different countries and continents [28, 109].

The serotype of *Y. enterocolitca* is always based on biochemical and genetic features ('O' or LPS). The LPS molecule is composed of two biosynthetic entities: the lipid A-core and the O-polysaccharide (O-antigen). O-antigen (O-ag) plays an important role in the innate immune system to host tissues and resisting to antimicrobial element [107]. Since the O-ags are highly variable and strongly immunogenic, several serotypes were established for *Y. enterocolitica* based on the variability [117]. Some studies also showed the development and validation of *Y. enterocolitica* serotyping by using PCR-based method [118]. The location and genetic organization of the O-ag clusters of the analyzed serotypes (O:8, O:3, O:9 and O:5) are presented in **Fig 2**. The genes *per*, *wzt*, *wbbU* and *wbcA* can be the characteristic genes for the serogroups O:9, O:5, O:3 and O:8.

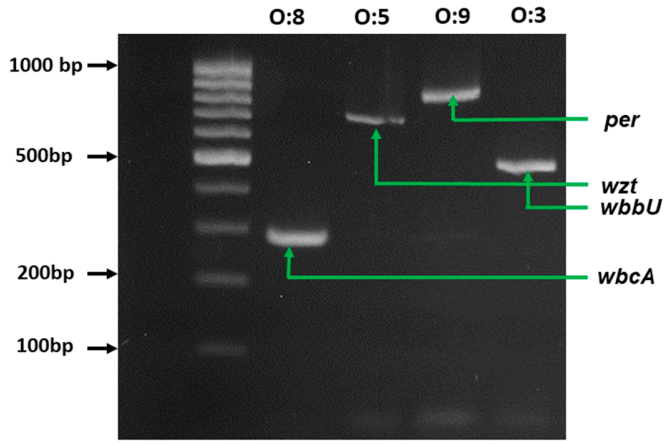

Fig 2: A molecular scheme for *Yersinia enterocolitica* serotyping.

Serotyping has been proved useful for characterization and surveillance of the bacteria. According to the several serotypes, **Table 7** shows the frequencies of the serotypes causing yersinoses in Germany from 2001 to 2016. The numbers are the total infections of the respective years with the associated incidence ever 100,000 inhabitants. **Fig 3** shows the relative frequency of the main serotypes (O:3, O:9, O:8 and O:5,27) from 2001 to 2016.

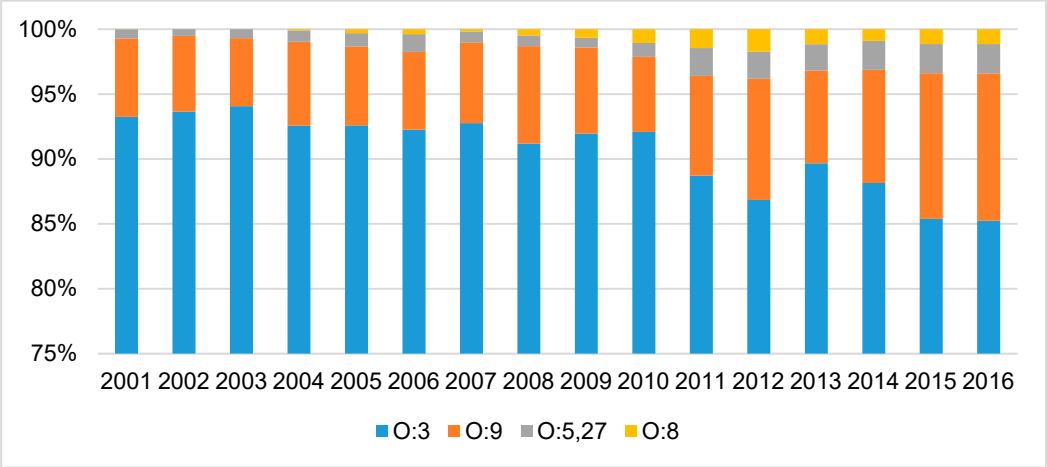

Fig 3: Relative frequency of *Y. enterocolitica* main serotypes (2001-2016)

Table 7: Frequency of *Y. enterocolitica* serotypes associated with human diseases in Germany [68-73]

Serotype	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016
O:3	4304	5227	4826	4672	4306	3945	3935	3361	2906	2594	2440	1868	1711	1659	2092	2080
O:9	279	325	269	325	282	256	264	277	209	163	211	201	136	164	275	277
O:5,27	32	29	35	44	48	59	35	30	24	30	59	45	39	42	55	55
O:8	1	0	0	5	14	16	8	18	21	30	40	37	22	17	28	28
Others	175	342	432	185	149	155	118	145	111	70	83	103	142	168	NA	NA
Unknown	2404	1617	1015	953	829	730	628	523	460	481	564	451	540	431	NA	NA
Total	7195	7540	6577	6184	5628	5161	4988	4354	3731	3368	3397	2705	2590	2485	2752	2774
Incidence	8.7	9.1	8	7.5	6.8	6.3	6.1	5.3	4.5	4.1	4.2	3.3	3.2	3.1	3.4	3.4

As demonstrated in **Table 7**, the total number of yersinoses cases and the incidence of *Y. enterocolitca* infections decreased over the entire period except for the year 2015 and 2016. From the reports of frequencies of main serotypes in **Table 7** and **Fig 3**, the infections caused by serotype O:3 strains decreased over the years. In contrast, infections by serotype O:8, O:9 and O:5,27 increased. Since the O:3 strains are less virulent and the diagnosis method developed these years, less human infection caused by O:3 might be reported less frequently [119]. However, in recent years, the infections with isolates of this serotype also occurred in Germany [68-73].

2.4.2 Biotype

According to the essential factor based on pathogenicity, *Y. enterocolitica* can be characterized in six biotypes (1 A, 1B, 2, 3, 4, and 5) [120]. Biotype 1A used to be reported as the non-virulent type. However, the pathogenic potential of Biotype 1A isolates is still under debate [121, 122]. Biotype 1A strains have been repeatedly isolated from patients with gastrointestinal symptoms though biotype 1A strains lack the pYV virulence plasmid [123]. In contrast, the biotypes 1B and 2-5 possess the *Yersinia* virulence plasmid (pYV) [124, 125].

Various biotypes and even more numbers of serotypes and bio/serotypes have been found according to their characteristics. Based on epidemiological reports, there is a mutual relationship between biotypes and serotypes. **Table 8** shows the combinations of biotypes and serotypes.

Table 8: Combinations between biotypes and serotypes [126]

Biotype	Serotype	Major Hosts	Human
1A	O:4; O:5; O:6,30, O:6,31; O:7,8; O:7,13; O:10; O:14; O:16; O:18; O:21; O:22; O:25; O:37; O:41,42; O:46; O:47; O:57; NT	Pork	-
1B	O:4,32; O:8; O:13a/b; O:16; O:18; O:20; O:21; O:25; O:41,42; NT	Pork (O:8)	+
2	O:5,27, O:9, O:27	Pork (O:9)	+
3	O:1,2,3, O:3, O:5,27	Pork (O:5,27)	+
4	O:3	Pork	+
5	O:2,3	Rabbit	+

Among all the common *Y. enterocolitica*, strains with the highest relevance to humans are O: 5,27, O: 8, O: 9 and O: 3. Most clinical isolates belong to bioserotypes (e.g., 4/O:3, 3/O:9 and 1B/O:8), which are also frequently isolated from food and animals [127]. In addition, bioserotypes are generally considered distributed according to different geography. For example, strain 1B/O:8 has been the predominant version of pathogenic *Y. enterocolitica* in the U.S. [128]; strain 3/O:9 is the common cause of Yersiniosis in China and Europe [129, 130]. Bioserotype 4/O:3 is the most important human pathogen in European countries and it is the predominant pathogenic bioserotype in slaughter pigs in Finland and Germany [131, 132]. Recently, bioserotyp 4/O:3 has also emerged as an important cause of Yersiniosis in the US.[133].

2.4.3 Virulence

Y. enterocolitica is an intracellular pathogen. It invades and survives within macrophages and may persist and grow within lymph nodes and other lymphoid tissue (in animals and in humans). Adherence, invasion and survival in lymphoid tissue depend on the chromosome virulence plasmid (pYV). A range of virulence factors are encoded by pYV: Ail (the attachment-invasion locus), YadA (Yersinia adhesion A) and lipopolysaccharides [23]. The changes in environmental and/or host conditions (including changes in temperature) can result in gene expressional changes, which are related to virulence. It was reported that *Y. enterocolitica* strains show a temperature responsive adaptation which aids the transition from environmental adaptation to within-host survival and host infection. This process determines the clinical outcome and the incubation period [28]. Thus, regulation of virulence plays a key role in the successful infection.

2.5 *Yersinia enterocolitica* at low temperature

2.5.1 Survival at cold temperature

As a psychrotrophic bacterium, *Y. enterocolitica* is able to survive both outside and inside hosts under varying temperatures [134-137]. They are capable of growing at temperatures approaching and even below 0 °C [129, 134, 138]. Therefore, refrigeration temperatures (0 – 4 °C) do not kill *Yersinia* strains and even ensure a significant rise of bacteria growth. At temperatures for food storage, several researchers have reported growth of *Y. enterocolitica*. *Y. enterocolitica* are able to multiply at refrigeration temperatures (0 °C and 2 – 4 °C) on e.g. raw bovine meat in certain cases with 2 log CFU/ml within 4 days [129]. In pasteurized milk held at 4 °C, *Y. enterocolitica* growth increased and reached levels of log 5.0 to 7.0 CFU/ml after 7 d [139]. The survival of this bacteria was reported in oysters stored at 0 - 2 °C for 14 - 21 d and at 5 - 7 °C for 2-10 d [140]. The highest prevalence of *Y. enterocolitica* in wild boars were observed during winter and spring time at the low temperature [82]. Similarly, in pigs slaughtered in China, the incidence of *Y. enterocolitica* was higher in cold than in warm areas [141].

2.5.2 Regulation of cold-induced protein

Bacteria respond to a rapid temperature downshift by triggering a physiological process to cope with stress and adapting to unfavorable conditions. The cold response leads to a growth acclimation and overall adjustment of translation. However, there is a set of specific proteins induced to tune cell metabolism and readjust it to the new conditions [142, 143]. They are cold-induced proteins (Cips). The increased production of Cips is related to the severity of the cold shock [144-146]. Most research on Clps is done in *E. coli* and numerous Cip related units have been identified so far, including the cold shock protein (Csp) family [147], RNA helicase [148] exoribonucleases PNPase and RNaseR [149] [150], initiation factors 2α, 2β, NusA and RecA [151-153]. **Table 9** focuses on the essential genes encoding Cips and their functions.

Table 9: Functions of the cold-induce genes

Gene	Function in cold shock	References
aceE	Pyruvate dehydrogenase, decarboxylase	[153]
aceF	Pyruvate dehydrogenase, dihydrolipoamide acetyltransferase	[153]
cspA	Cold-inducible RNA chaperone and anti-terminator; transcriptional enhancer	[153]
cspB	Cold shock-inducible; function unknown	[153]
cspE	RNA chaperone; transcriptional antitermination	[153]
cspG	Cold shock protein homologue, cold-inducible; function unknown	[153]
cspI	Cold shock protein, cold shock-inducible; function unknown	[153]
csdA	Cold-shock RNA helicase, related to the biogenesis of 50S ribosomal subunit	[148]
deaD	ATP-dependent RNA helicase, facilitates translation of mRNAs	[154, 155]
dnaA	DNA binding and replication initiator, global transcription regulator	[153]
gyrA	DNA gyrase, subunit A; DNA binding/cleaving/rejoining subunit of gyrase	[153]
hns	Nucleoid protein, transcriptional repressor, repressor supercoiling	[153]
hscA	DnaK-like chaperone	[156]
hscB	DnaJ-like co-chaperone for HscA	[156]
hupB	Nucleoid protein, DNA supercoiling	[157]
infA	Protein chain initiation factor IF1, translation initiation	[153]
infB	Protein chain initiation factor IF2, translation initiation, fMet-tRNA binding, protein chaperone	[153]
infC	Protein chain initiation factor IF3, translation initiation, stimulates mRNA translation	[153]
lpxP	Lipid A synthesis; cold-inducible	[158, 159]
nusA	Transcription termination/antitermination/elongation L factor	[151]
otsA	Trehalose phosphate synthase; cold- and heat-induced, critical for viability at low temperatures	[160]
otsB	Trehalose phosphate phosphatase; cold- and heat-induced, critical for viability at low	[160]
pnp	exoribonuclease; component of RNA degradosome; cold shock protein required for growth at low temperatures	[149]
rnr	exonucleases; increases 10-fold in cold shock	[150]
rbfA	Ribosome-binding factor required for efficient processing of 16S rRNA; cold shock adaptation protein	[153]
recA	General recombination and DNA repair; induction of the SOS response	[152, 153]
tig	Protein-folding chaperone, multiple stress protein, ribosome-binding	[160, 161]
ves	Cold- and stress-inducible protein, function unknown	[162]
yfiA	Protein Y, associated with 30S ribosomal subunit, inhibits translation	[152]

As a kind of critical Cip, Cold shock proteins (Csp) play a decisive role in response to cold. They have been found in psychrophilic, mesophilic, thermophilic and even hyper thermophilic bacteria [145, 163]. The classification and the function of cold shock proteins have been mostly reported in *E. coli*. Within this protein family, CspA was the major cold shock protein firstly described in *E. coli*. It is the first protein that is induced after a downshift in temperature account for the 13% of the total protein synthesis [164]. In addition to CspA, nine homologous proteins sharing 46–91% amino acid sequence similarity were identified in *E. coli* [165]. Only CspA, CspB, CspE, CspG and CspI of *E. coli* are induced by cold [166-170]. According to the published genome sequences of *Yersinia* spp, 10 *csp* genes were identified in *Y. enterocolitica* strain 8081 [171], 10 *csp* genes in *Y. pseudotuberculosis* strain IP32953 [172] and 8 *csp* genes in *Y. pseudotuberculosis* strain IP31758 [173]. According to other reports, only *cspA1* and *cspA2* of *Y. enterocolitica* have been well investigated [174, 175]. However, no information exists on the indispensability or role of individual *csp* genes or on their regulation in enteropathogenic *Yersinia*.

RNase E and PNPase are proposed to cleave *csp* transcripts endonucleolytically within CSC boxes [176]. Accordingly, PNPase is essential for *Y. enterocolitica* and *E. coli*, and it contributes to selective degradation of Csp mRNAs [176-179]. As we mentioned before, during the cold response, the synthesis of Cips can help the cells adapt to lower temperatures. However, research on the exact function of Cips is still rare.

2.6 General information on proteomic analysis

Many scientists around the world improved the methods of sequencing and analyzed many genomes in order to know the organization of life and the regulatory networks. The basic knowledge and methodologies to elucidate functional regulatory networks on protein level are more important than DNA-based discovery tools. This was mainly due to the fact that proteins have universal functions not only in amino acid sequences but also in their various shapes, sizes and physical and chemical properties. In addition, structure, function and the respective stability of proteins of a biological system allow the organism adaptation to any environment [180].

Over the last ten years, proteomic technology for microorganisms developed and was used in biotechnological products and processes. For instance, sodium dodecyl sulfate polyacrylamide protein electrophoresis (SDS-PAGE) is the common gel-based methods used with the strong detergents allowing the solubilization of most proteins. Proteins are separated according to their size and can be identified by mass spectrometry (MS). The method is inexpensive and well suited for preliminary and general research. Meanwhile, the low resolution capability of SDS-PAGE is the major shortcoming, which limits the confident protein identification [181-183]. Now, 2-DE (2-dimensional polyacrylamide gel electrophoresis) combined with mass spectrometry are more widely used in the protein quantification. Recently, peptide mass fingerprinting and tandem mass spectrometry (MS/MS) are relied on making such identification fairly easier [184, 185]. Methods have been developed, such as multidimensional liquid chromatography (LC) for protein separation and MS for protein identification and the LC-MS approaches have become proteomics procedures relying on more-sophisticated equipment [186]. ‘

Rapid development of the gel-free methods with the lower amount of sample and less complex of peptide mixture and bioinformatics technologies provides an essential approach to investigate whole variations in protein expression. Especially in the aspect of high-throughput comparative proteomics, it enables the parsing of various potential mechanisms and regulatory networks in bacteria [187]. As the most popular isotope labeling methods, iTRAQ (isobaric tag for relative and absolute quantitation) present different isobaric tags that bond to N-terminus, lysine residues and side chain amine peptides [188]. Recently, the application of iTRAQ in various stress response networks and functional mechanisms has been applied in many bacteria. Label-free methods arise from the necessity to overcome some prime limitations of labeling methods. Besides, it requires a smaller amount of sample and allows multiproteome analysis within the same experiment [187]. This method has also been used for multiple stress response in bacteria. It was reported that this quantitative analysis of *Brucella abortus* revealed metabolic adaptation to various environmental stresses including nutrient limitation, low pH, antimicrobial defenses, and reactive oxygen species (ROS) via the host immune response [189]. In *Y. ruckeri*, the label-free proteomic analysis was settled under iron-limited conditions. Sixty-one differentially expressed proteins were identified involved in processes including iron ion capture and transport and enzymatic metabolism [190].

During the development of proteomics overtime, cold response in protein level was deeply investigated in many organism and with various methods. Comparative antigenic proteins and proteomics were compared in *Y. enterocolitica* under different temperatures [191]. However, to our knowledge, no study has been carried out on the global proteomic profile of *Y. enterocolitica* induced by low temperature. Therefore, label-free quantitative proteomic analysis was applied to investigate the proteomic changes in response to cold of *Y. enterocolitica* in this study. In order to explain the biological mechanism of the cold response and resistance of *Y. enterocolitica*, the global proteomic analysis and the cold differential phenotypes were detected in isolates to achieve an in-depth understanding of stress responses. It will be helpful to predict microbial fate when they encounter cold temperatures and to design and develop more effective strategies to control pathogens in food for ensuring food safety.

CHAPTER 3 PREVALENCE AND ANTIMICROBIAL RESISTANCE OF *YERSINIA ENTEROCOLITICA* IN RETAIL SEAFOOD

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CHAPTER 4 ISOLATE SPECIFIC COLD RESPONSE OF *YERSINIA ENTEROCOLITICA* IN TRANSCRIPTIONAL, PROTEOMIC, AND MEMBRANE PHYSIOLOGICAL CHANGES

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Isolate Specific Cold Response of *Yersinia enterocolitica* in Transcriptional, Proteomic, and Membrane Physiological Changes

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Yersinia enterocolitica, a zoonotic foodborne pathogen, is able to withstand low temperatures. This psychrotrophic ability allows it to multiply in food stored in refrigerators. However, little is known about the *Y. enterocolitica* cold response. In this study, isolate-specific behavior at 4°C was demonstrated and the cold response was investigated by examining changes in phenotype, gene expression, and the proteome. Altered expression of cold-responsive genes showed that the ability to survive at low temperature depends on the capacity to acclimate and adapt to cold stress. This cold acclimation at the transcriptional level involves the transient induction and effective repression of cold-shock protein (Csp) genes. Moreover, the resumption of expression of genes encoding other non-Csp is essential during prolonged adaptation. Based on proteomic analyses, the predominant functional categories of cold-responsive proteins are associated with protein synthesis, cell membrane structure, and cell motility. In addition, changes in membrane fluidity and motility were shown to be important in the cold response of *Y. enterocolitica*. Isolate-specific differences in the transcription of membrane fluidity- and motility-related genes provided evidence to classify strains within a spectrum of cold response. The combination of different approaches has permitted the systematic description of the *Y. enterocolitica* cold response and gives a better understanding of the physiological processes underlying this phenomenon.

Keywords: *Yersinia enterocolitica*, cold response, proteome, isolates specific, motility, fluidity

INTRODUCTION

Yersinia enterocolitica, the third most commonly reported foodborne zoonotic pathogen in the European Union, can cause serious diseases, including gastroenteritis, mesenteric lymphadenitis, reactive arthritis, erythema nodosum, and pseudoappendicitis (Ostroff et al., 1994; Horisaka et al., 2004; European Food Safety Authority and European Centre for Disease Prevention and Control, 2016). It occurs ubiquitously in the natural environment and is widespread in animal populations (Benembarek, 1994; Robins-Browne, 2013). Furthermore, it can be isolated frequently

from a variety of foods, including milk and milk products, pork, poultry, eggs, and produce (Bari et al., 2011).

Yersinia enterocolitica is capable of growing at temperatures approaching and even below 0°C (Tudor et al., 2008; Divya and Varadaraj, 2013). Therefore, even refrigeration temperatures (0–4°C) can allow significant bacterial growth over time. Several studies have reported growth of *Y. enterocolitica* in food products stored at refrigeration temperatures: e.g., on raw beef, with increased cell counts of up to 2 log CFU/ml within 4 days (Tudor et al., 2008) and in pasteurized milk, reaching levels of 5–7 log CFU/ml after 7 days (with an initial inoculum of 1–3 log CFU/ml) (Amin and Draughon, 1987).

One of the most prominent cold responses is the induction of cold-shock proteins (Csps) in all psychrotrophs, mesophiles, and thermophiles (Polissi et al., 2003; Phadtare, 2004). As model systems, *Escherichia coli* and *Bacillus subtilis* have been studied in detail regarding cold response and Csps (Phadtare et al., 1999; Ermolenko and Makhatadze, 2002; Weber and Marahiel, 2003). The role of polynucleotide phosphorylase (PNPase, encoded by the *pnp* gene) in regulating cold response is also well described (Goverde et al., 1998; Yamanaka and Inouye, 2001; Cordin et al., 2006; Matos et al., 2009; Phadtare, 2011). This enzyme with the 3'- to 5'-exonucleolytic activities involved mostly in mRNA decay and ribosomes release (Coburn and Mackie, 1998; Polissi et al., 2003) is used to help repress the generation of Csps and relieve growth arrest (Neuhaus et al., 2003; Zhao et al., 2016). Meanwhile, in psychrotrophic bacteria such as *Arthrobacter globiformis* and *Pseudomonas fragi*, some cold-responsive proteins are synthesized at relatively moderate levels and prolonged in response to continuous growth at low temperatures (Berger et al., 1996; Michel et al., 1997). These proteins are of particular importance since they differentiate psychrotrophs from mesophiles, and they are probably one of the key determinants that allow survival at low temperature (Hébraud and Potier, 1999). Additionally, the ability to cope with temperature downshift must be accompanied by a number of changes in response to alterations of physical and biochemical parameters, including solubility, membrane fluidity, protein conformation and stability, and changes in gene expression (Hébraud and Potier, 1999; Vorachek-Warren et al., 2002; Albanesi et al., 2004; Phadtare, 2004; Cao-Hoang et al., 2010; Barria et al., 2013). Therefore, the biochemical and physiological effects allowing bacteria to adapt to temperature changes are likely to be complex, involving a number of cellular processes.

As a psychrotrophic bacterium, *Y. enterocolitica* has two well reported *csp* homolog genes (*cspA* and *cspB*), which are strongly expressed during the cold response. The cold-shock exoribonuclease PNPase and *pnp* gene have also been reported (Goverde et al., 1998; Phadtare, 2011). Additionally, a previous study has reported that genes involved in various functions (regulation, motility, virulence, and metabolism) are upregulated after a temperature downshift from optimal (30°C) to suboptimal (10°C) conditions in *Y. enterocolitica* (Bresolin et al., 2006). However, the effects of these genes and the cold response on protein expressional levels are not clarified in *Y. enterocolitica*.

Recently, advances in proteomics and bioinformatics technologies provide clear information on protein expression in response to cold and other stresses. High-throughput comparative proteomics with label-free quantification enabled the parsing of various potential mechanisms and regulatory networks of stress response in *E. coli*, *B. subtilis*, *Pseudomonas putida*, and *Yersinia ruckeri* (Delumeau et al., 2011; Stefanopoulou et al., 2011; Herbst et al., 2015; Kumar et al., 2016).

However, to our knowledge, the global proteomic profiles of *Y. enterocolitica* under the influence of low temperature have not been reported. Considerable research on *Y. enterocolitica* cold response has been limited to few proteins or genes and to single time points. The aim of this study is to describe the physiological processes of cold response in *Y. enterocolitica* via comparisons of growth ability, expression of cold-responsive genes and proteins, as well as cell motility and membrane fluidity of selected strains upon exposure to cold conditions.

MATERIALS AND METHODS

Growth Profile at Low Temperature

In order to test the growth ability of *Y. enterocolitica* at low temperatures (4°C), 55 isolates were collected from different matrices, representing different serotypes and biotypes (details are given in **Table 1**). Isolates were incubated on Plate Count agar (PC agar, Merck, Darmstadt, Germany) at 28°C for 24 h. Single colonies were transferred to 3 ml of *Brucella* broth (BB, BD Franklin Lakes, NJ, United States) and incubated at 28°C for 20 h. Enriched cultures were serially diluted 1:10^6 in BB to reach a cell concentration of about 10^1–10^2 CFU/ml as the initial value. Growth abilities of 55 strains were tested based on cell concentration in BB after incubating at 4°C for 168 h. For growth profile investigation, cell concentration of the selected isolates (II7D, 8081, and 44B) was measured under cold stress for 0, 24, 48, 72, 144, and 168 h respectively. The experiment was carried out in six biological replicates (with two technical duplicates each).

RNA Extraction Under Cold Stress

Yersinia enterocolitica isolates were selected for RNA extraction. Pre-culture was prepared in 12 ml BB at 28°C (as incubation temperature) for 24 h. The suspension was diluted in BB to 0.05 OD_{600} value and then incubated at 28°C for 2 h to reach an OD_{600} value between 0.1 and 0.2. After centrifugation, the bacteria were suspended into 10 ml cooled BB and incubated at 4°C for different time periods (5 min, 30 min, 2 h, 4 h, 24 h, and 48 h). The pellet suspended in BB at room temperature was used as control. Cold-shock stop mix solution (5% Roti-Aqua-phenol, 95% ethanol, Carl Roth, Karlsruhe, Germany) was added and samples were processed as described elsewhere (Blomberg et al., 1990). All samples were frozen at −80°C until further use.

RNA was extracted with Roti-Aqua-Phenol (Carl Roth). RNA quality of samples was tested by gel electrophoresis. The ratio of absorbance A_{260}/A_{280} and A_{260}/A_{230} were used to assess the purity of RNA photometrically with NanoDrop™ 2000/2000c Spectrophotometers (Thermo Fisher Scientific). A ratio of ∼2.0 is generally accepted of A_{260}/A_{280} and the expected A_{260}/A_{230} values are set in the range of 2.0–2.2. Reverse transcription

TABLE 1 | Characteristics and growth ability of *Y. enterocolitica* strains at 4°C for 168 h.

Isolates	Median	Median norm.	Serotype	Biotype	Matrix
44B	1.86E + 03	2.86E + 02	O:5,27	1A	Food
IP566/82	4.60E + 03	8.85E + 02	O:8	n. a.	n. a.
4780	5.90E + 05	5.98E + 04	O:8	1B	Human
96/10	1.50E + 05	1.01E + 05	O:8	1B	n. a.
39/91	5.00E + 06	2.87E + 05	O:8	1	Human
21/08	4.20E + 07	3.41E + 06	O:8	1A	n. a.
8081	2.70E + 07	6.85E + 06	O:8	1B	Human
78/90	1.07E + 08	7.96E + 06	O:8	1B	Human
25 Ia	1.25E + 08	1.22E + 07	O:3	4	Food
96B	2.60E + 08	2.23E + 07	O:5,27	3	Animal
177B	2.40E + 08	2.37E + 07	O:5,27	2	Human
207 IIa	3.60E + 08	2.64E + 07	O:9	3	Animal
207 Ia	3.40E + 08	3.56E + 07	O:9	3	Animal
25/13	8.10E + 08	4.60E + 07	O:5	1A	Food
56/14	5.90E + 08	4.66E + 07	O:5	1A	Food
57/14	7.40E + 08	4.76E + 07	O:9	2	Food
54/13	6.45E + 08	4.93E + 07	O:8	1A	Food
28/07	1.00E + 09	5.03E + 07	O:9	3	Animal
04/13	4.90E + 08	5.05E + 07	O:5	1A	Food
32/07	4.30E + 08	5.77E + 07	O:3	4	Animal
05/13	5.80E + 08	5.79E + 07	O:5	1A	Food
44/07	1.14E + 09	5.92E + 07	O:3	4	Food
III15D	7.00E + 08	5.93E + 07	O:5	1A	Food
24/14	4.50E + 08	5.97E + 07	O:5	1A	Food
29/07	8.30E + 08	5.99E + 07	O:9	3	Animal
09/11	1.37E + 09	6.12E + 07	O:9	2	Food
37/12	5.40E + 08	6.18E + 07	O:5	1A	Food
65/14	1.03E + 09	6.29E + 07	O:5	1A	Food
47/13	5.50E + 08	6.31E + 07	O:5	1A	Food
77/14	6.40E + 08	6.46E + 07	O:9	2	Food
I15C	6.80E + 08	6.92E + 07	O:3	3	Animal
20/07	1.42E + 09	7.17E + 07	O:9	3	Human
11/07	1.07E + 09	7.32E + 07	O:3	4	Human
38/12	5.60E + 08	7.35E + 07	O:5	1A	Food
58/07	1.13E + 09	7.73E + 07	O:3	4	Animal
31/13	7.60E + 08	7.86E + 07	O:5,27	2	food
45/14	1.21E + 09	7.97E + 07	O:5,27	2	Food
03/13	7.00E + 08	8.01E + 07	O:8	1A	Food
387/09	8.20E + 08	8.06E + 07	O:9	n. a.	Animal
06/13	4.30E + 08	8.36E + 07	O:8	1B	Food
18/07	1.30E + 09	8.45E + 07	O:9	3	Human
15/12	9.40E + 08	8.92E + 07	O:5,27	2	Food
19/07	1.07E + 09	9.62E + 07	O:3	4	Human
61/07	1.12E + 09	1.00E + 08	O:3	4	Animal
13/14	1.12E + 09	1.01E + 08	O:3	4	Food
12/07	8.80E + 08	1.02E + 08	O:3	4	Human
30/14	9.50E + 08	1.04E + 08	O:5,27	2	Food
33/07	4.50E + 08	1.09E + 08	O:3	4	Animal
25/14	8.80E + 08	1.12E + 08	O:5,27	2	Food
11/09	1.07E + 09	1.14E + 08	O:5,27	2	Food
14/07	1.45E + 09	1.15E + 08	O:3	4	Human
46/14	1.56E + 09	1.21E + 08	O:5,27	2	Food
89/14	1.42E + 09	1.27E + 08	O:3	4	Food
17/07	9.70E + 08	1.27E + 08	O:9	3	Human
II7D	1.02E + 09	1.27E + 08	O:5	1A	Food

Median norm.: relative median value normalized with the initial concentration, respectively.

was performed with Maxima H Minus First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). The cDNA samples were diluted 1: 5 with nuclease-free water for RT-qPCR investigation.

Expressional Analysis of Cold-Responsive Genes

Real-time quantitative PCR (RT-qPCR) was used to test the transcription level of cold-responsive genes of *Y. enterocolitica*. Eight genes, which were reported to have enhanced at transcriptional levels at 10°C (Bresolin et al., 2006), were tested in this study. These genes cover the functions of regulation, metabolism, and motility (**Supplementary Table S2** lists target genes and used primers). The SsoFast EvaGreen Supermix (SYBR-green, Bio-Rad, Munich, Germany) was used for RT-qPCR assays. The expression of the genes was normalized to the reference gene *polA* (DNA polymerase I) (Townsend et al., 2008). The results of RT-qPCR were visualized and evaluated by CFX software (Bio-Rad).

Whole Cell Protein Extraction

Three isolates (*Y. enterocolitica* strains II7D, 8081, and 44B) were subjected to incubation at 4°C for 0, 5 min, 2 h, and 24 h. The cells were harvested and the pellet was washed with PBS. Cell pellets were reconstituted with 300 μl distilled water and inactivated by addition of 900 μl ethanol. After the centrifugation and evaporation, the final pellet was reconstituted with 250 μl 20 mM HEPES (pH 7.4) and subjected to sonication for 1 min (cycle, 1.0; amplitude, 100%) with a sonicator (UP100H; Hielscher Ultrasound Technology, Teltow, Germany). Supernatants were collected and the concentration was measured using modified Bradford's method with Coomassie Plus™ Protein Assays (Thermo Fisher Scientific, Rockford, IL, United States) and the samples were stored at -20°C for further analysis. Each strain was tested six times independently.

In-Solution Trypsin Digestion

The *in-solution* trypsin digestion of proteins was performed as described previously (Wareth et al., 2016). Briefly, 10 μg protein was used for acetone precipitation. The resultant peptides were then reconstituted with 20 μl denaturation buffer containing 6 M urea/2 M thiourea in 10 mM HEPES (pH 8.0) and reduced with 10 mM dithiothreitol in 50 mM of ammonium bicarbonate (ABC, Sigma, Germany). The alkylation was carried out with 55 mM iodacemtamide and subsequently 0.5 μg/μl LysC solution was added. The urea concentration was decreased by 0.5 μg/μl trypsin and the trypsin digestion was stopped by 5% acetonitrile/3% trifluoroacetic acid.

Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry (LC-ESI-MS/MS) Measurements

Liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS/MS) measurements were carried out as described elsewhere (Wareth et al., 2016). Resultant peptides of trypsin digestion were desalted by solid phase extraction and the peptides were separated using Dionex Ultimate 3000

nanoLC (Dionex/Thermo Fisher Scientific, Idstein, Germany) on fritless silica micro-columns with an inner diameter of 100 μm. Mass spectrometry measurements were carried out using LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The LTQ-Orbitrap was operated in the positive mode to simultaneously measure full scan MS spectra in the range of *m/z* 300–1700 in the Orbitrap analyzer at a resolution of $R = 60{,}000$. After that, isolation and fragmentation of the 20 most intense ions in the LTQ part were carried out by collision-induced dissociation.

The raw mass spectra were processed using label-free quantification algorithm of the MaxQuant version 1.3.0.5 (Max Planck Institute of Biochemistry, Martinsried, Germany) (Tyanova et al., 2016) and protein identification was carried out by searching against protein sequence FASTA file of *Y. enterocolitica* strain YE02/02 (Proteome ID: UP000069750, protein count: 4760) with a wide range of homologous strains downloaded from UniProt database. The following parameters were set for protein identification: Initial maximum precursor—7 ppm, fragment mass deviations—0.5 Da; variable modification—methionine oxidation/acetylation of peptide N-termini; fixed modification—carbamidomethylation; enzymes—LysC and trypsin, both with a maximum of two missed cleavages; minimum peptide length—seven amino acids, and target-decoy-based false discovery rate (FDR) for peptide and protein identification—1%.

The statistical analysis was performed using the Perseus software version 1.4.1.3 (Max Planck Institute of Biochemistry, Martinsried, Germany) (Rudolph and Cox, 2019). The LFQ intensities of proteins were imported and transformed to logarithmic scale with base two. The Student's *t*-test and Benjamini–Hochberg procedure FDR corrections of the significant *p*-values ($p < 0.05$) were applied for identification of differentially expressed proteins.

Motility Assay

Motility was tested as described for *Y. enterocolitica* (Bresolin et al., 2008). Three strains II7D, 8081, and 44B were assessed by measuring diameters of migration zone at 4°C with motility agar plates (0.3% agar, 0.5% NaCl, and 1% tryptone). Strains were incubated on PC agar plates overnight at 28°C. Single colonies were transferred onto motility agar plates and incubated initially at 37°C for 2 h to start the assay with non-motile bacteria. Plates were subsequently incubated at 28°C (for 21 h) and 4°C (for 44 h).

Fluidity Assay

Membrane fluidity of *Y. enterocolitica* was measured by a fluorescence polarization or anisotropy value, which corresponds to the reaction to polarized light of a fluorescent probe inside the membrane (Zaritsky et al., 1985; Aricha et al., 2004; Mykytczuk et al., 2007). Briefly, three isolates (*Y. enterocolitica* strains II7D, 8081, and 44B) were prepared and incubated at 4°C for 0, 2, 24, and 48 h with the method described above. Cultured cells were harvested and washed twice with PBS (10 mM, pH 7.4, Merck) and then incubated with 5 μM 1,6-diphenyl-1,3,5-hexatriene (DPH, Sigma–Aldrich, St. Louis, MO, United States) at 37°C for 1 h. Unlabeled cells were used as a scattering reference. The fluorescence polarization was measured using a Cary Eclipse Fluorescence spectrophotometer with Manual Polarizer (Agilent, Santa Clara, CA, United States) at 360 nm excitation and 430 nm emission. Fluorescence anisotropy was calculated by the formula $A = [I_{VV} - I_{VH}\ (I_{HV}/I_{HH})]/[I_{VV} + 2I_{VH}\ (I_{HV}/I_{HH})]$, where I is the corrected fluorescence intensity, and the subscripts V and H indicate the values obtained with vertical or horizontal orientations, respectively. The emission polarized filter was set either in the vertical (I_{VV}) or horizontal (I_{VH}) position. Decrease in fluorescence anisotropy reflected increases in the fluidity of the lipid bilayer, which controls or alters the mobility of DPH in the membrane.

Bioinformatics and Statistical Analysis

Cell counts of the growth assays were expressed as the median with range for all the isolates (CFU/ml) and other quantitative data were expressed as the mean with the standard error of the mean. Paired sample *t*-tests were applied to determine differences in growth profile, gene expression, and fluidity. GraphPad Prism 6 was used to carry out the analyses cited above.

The Gene Ontology (GO) database[1] and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database[2] were used to classify proteins and related pathways of proteins (Kanehisa et al., 2016). The Clusters of Orthologous Groups (COGs) functional categories of differentially expressed proteins were assigned by BLAST and searched with the COG database[3] referring to other research (Tatusov et al., 2001; Galperin et al., 2014).

RESULTS AND DISCUSSION

Growth Profiles of Isolates at Low Temperatures

Altogether, 55 isolates of *Y. enterocolitica* collected from food, humans, and animals were tested for their growth profiles at 4°C after 168 h (end-point analysis). Diverse growth abilities at 4°C among the isolates were observed. Most of the isolates displayed enhanced growth rates at 4°C over 168 h, up to 10^8 CFU/ml (23.63%) and 10^7 CFU/ml (61.81%), while a minority of strains (14.6%) showed a slighter increase, up to 10^2–10^6 CFU/ml (**Table 1**). More than 85% of tested strains exhibited enhanced growth rates, which indicated a general survival and growth ability of *Y. enterocolitica* at low temperatures. This result is consistent with the observations of high levels of this bacterium in food products; e.g., meat, milk, cheese, and oysters (Peixotto et al., 1979; Greenwood et al., 1985; Amin and Draughon, 1987; Wang et al., 2009), and natural environmental conditions; e.g., soil and aqueous at low temperature (Asadishad et al., 2013). However, significant differences in growth ability among the tested isolates were observed at 4°C, which demonstrates the growth specificity of isolates at low temperature. Similar

[1]http://www.geneontology.org

[2]http://www.genome.jp/kegg/pathway.html

[3]http://www.ncbi.nlm.nih.gov/COG/

observations (specific behavior of strains under low temperature) were found in *Y. enterocolitica* previously. For example, strains with various serotypes survived differently at 4°C in soil and river water (Tashiro et al., 1991). The impact of low temperatures on the survival of *Y. enterocolitica* strains differs when inoculated on raw pork samples at 4 and −20°C for 90 days (Iliev and Najdenski, 2008).

To investigate the cold response of *Y. enterocolitica*, three isolates [44B (1A/O:5,27), 8081 (1B/O:8), and II7D (1A/O:5)] representing low, medium, and high growth ability at 4°C, respectively, were selected for further analysis (**Supplementary Table S1**).

Transcriptional Changes of Cold-Responsive Genes at Low Temperature

In order to better understand the cold response in *Y. enterocolitica*, the correlation between growth ability and transcriptional changes was investigated in the three isolates. It has been mentioned that *pnp* gene played an indispensable role in the cold response of *Y. enterocolitica* (Goverde et al., 1998) and other bacteria (Mathy et al., 2001; Hu et al., 2014; Briani et al., 2016). In our study, during a cold response, an increased expression of *pnp* gene was detected (**Figure 1A**). When exposed to 4°C for 5 min to 2 h, the *pnp* expression of the three isolates exhibited no significant difference. After 4 h of exposure at 4°C, the expression of *pnp* in 44B increased continuously and significantly exceeded that of II7D and 8081. The results indicated that different changes of *pnp* expression were found among tested isolates with various growth ability, which verified the essentiality of the *pnp* gene in cold adaptation. The continuous high expression of *pnp* gene implies the higher demand of PNPase and *pnp* in 44B.

Based on the role of PNPase (encoded by the *pnp* gene) in repressing the generation of Csps and relieving growth arrest (Neuhaus et al., 2003; Zhao et al., 2016), the changes in related genes were investigated. RT-qPCR was performed with eight genes, which were reported to have increasing peaks or steady enhancement in gene expression after temperature downshift (Bresolin et al., 2006). The RNA used for this analysis was extracted from isolate cultures kept at 4°C from 5 min to 48 h and the related genes in response to cold with various functions are listed in **Supplementary Table S2** accordingly.

As **Figure 1B** shown, the expression of the genes *cspA*, *cspB*, *gltP*, and *uhpC* increased rapidly after a cold stimulation and then decreased over time, which is consistent with the result from previous study regarding changes of the cold-shock genes (Bresolin et al., 2006; Horn et al., 2007). Based on the expression of these cold-shock genes, the expression decreased rapidly in strains II7D and 8081 after the transcriptional peak. However, in strain 44B, the expression of these genes decreased slowly and the relative expression of *cspB*, *gltP*, and *uhpC* was higher than that of II7D and 8081 at the end of 4 h after cold stress. Since the function of PNPase was RNA degradation and the higher expression of *pnp* was observed in 44B (**Figure 1A**), the repression of Csp generation might not be accomplished in 44B.

As reported previously, after the repression of Csp production, the growth reinitiated at the end of the acclimation phase (Yamanaka and Inouye, 2001). Therefore, the RNA degradation of Csps by PNPase was indispensable for cold acclimation and growth resumption. Similar cold acclimation was also found in *E. coli*, in which the synthesis of Csps transiently increases and the control of mRNA stability and translatability plays a major role in the adaptive response to cold temperature (Phadtare et al., 1999; Briani et al., 2016).

A different cold response was detected on transcriptional levels of YE1436, *fleC*, *fliS*, and YE2848, which did not show increased peaks but mostly increased under cold stress over prolonged growth. According to the expression of genes YE1436 and YE2848, the transcriptional levels increased over time and the upward tendencies in II7D and 8081 are more obvious than that in 44B (even no obvious uptrend of YE1436 gene expression). After 48 h of cold stress, the relative expression of YE1436 and YE2848 was significantly lower in 44B compared with II7D and 8081. Considering the worse growth ability of 44B at low temperature, the transcriptional regulation of gene YE1436 and YE2848 might be necessary for cold response. As it was mentioned in other studies, one of the psychrotrophic abilities in bacteria was to produce several non-Csps and allow growth during prolonged low temperatures in cold adaptation (Berger et al., 1996; Hébraud and Potier, 1999; Wouters et al., 2000; Phadtare, 2004). After the cold acclimation, the expression of non-cold shock genes has not been resumed in 44B, arresting the transition from acclimation to cell growth.

In addition, after 48 h of cold stress, the expression of *fleC* and *fliS* genes increased in 44B while their expression did not increase until 24 h in II7D and 8081. Since the genes *fleC* and *fliS* are associated with bacterial motility, the regulation of motility might contribute to cold adaptation as well.

Consequently, the transcriptional changes in cold-responsive genes play an important role in both cold acclimation and prolonged adaptation. The isolate-specific ability to survive under cold stress depends on the capacity of enabling transient induction and effective repression of cold-shock gene in cold acclimation. Meanwhile, the resumption of the non-cold shock gene expression was also required in prolonged cold adaptation.

Global Proteomic Analysis of the Cold-Responsive Proteins at Low Temperature

Three isolates (II7D, 8081, and 44B) with various growth abilities were used for the proteomic analysis to further investigate the underlying processes of cold response. A total of 1526 proteins were identified using label-free quantification analysis in six biological replicates. Among these, 809 proteins which expressed differentially under cold stress (at 4 versus 28°C) for 5 min, 2 h, and 24 h in three strains were identified. Functional classification and annotation indicated that 715 and 790 uniproteins were assigned to 30 GO annotations and 138 KEGG functional pathways, respectively.

The proteins assigned to GO functional groups were classified into three categories: "biological process," "molecular

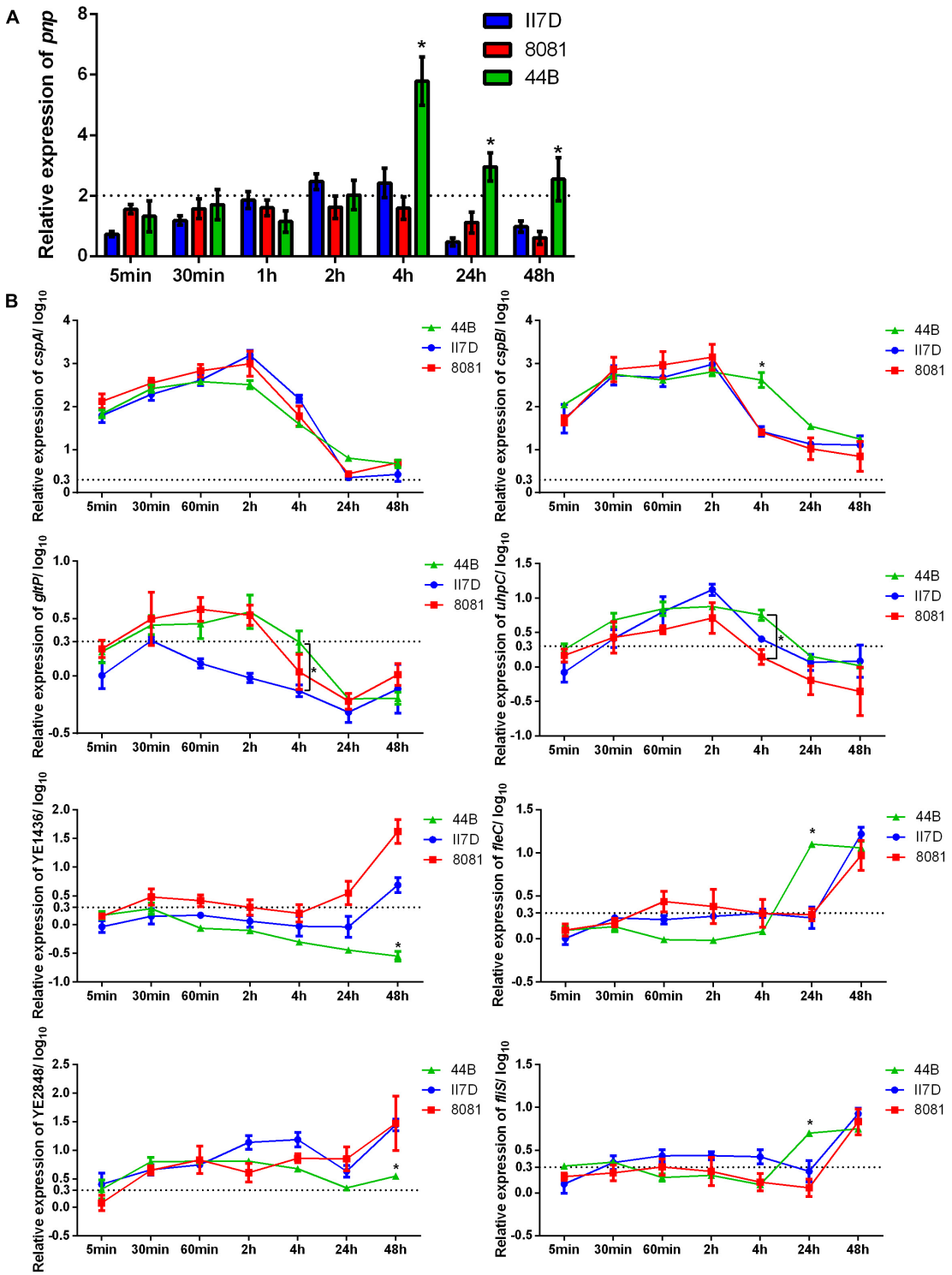

FIGURE 1 | Expressional changes of the cold-responsive genes in II7D, 44B, and 8081. Three *Y. enterocolitica* strains were incubated at 4°C over time (from 5 min to 48 h) to show the expressional changes in cold response. Gene expression was normalized to the reference genes *polA*. **(A)** Expressional changes of *pnp* gene of three isolates at 4°C. Specific values are shown as the means ± SEM of the relative expression in four independent experiments. **(B)** Expressional changes of *cspA*, *cspB*, *gltP*, *uhpC*, YE1436, *fliS*, *fleC*, and YE284 genes at 4°C in three isolates. Specific values of relative gene expression are shown in $\log_{10}$ as the means ± SEM of four independent experiments. Statistically significant difference compared with the control according to multiple comparisons (*$p < 0.05$). The line parallel to the *x*-axis represents a biologically relevant induction at 2 (fold-change) and 0.3 (log10 fold-change).

function," and "cellular component" (**Figure 2**). Various biological processes were involved in cold response. The most predominant processes were cellular and metabolic process; other major process categories were biological regulation, localization, and cellular component organization or biogenesis. These results indicated that the effects of cold response on protein level in *Y. enterocolitica* were involved in multiple processes. Furthermore, the predominant molecular functions of expressed proteins were associated with catalytic activity and binding; molecular functions of transporter and structural molecule activity were also involved in. Additionally, the most predominant cellular components were located cell and membrane parts. These results implied that the metabolism of the bacteria changed severely after cold response and it might lead to the alterations of cell and membrane components. Considerable groups of temperature-associated proteins were also reported previously in many other studies. For example, the periplasmic proteins associated with cellular component organization are strongly altered in *Yersinia pestis* in response to temperature changes (Pieper et al., 2008). The proteins involved in metabolic processes highly expressed at 4°C in *Listeria monocytogenes* (Cacace et al., 2010). During an abrupt temperature downshift in *E. coli*, expressional alterations occurred in the proteins associated with transport and binding (Kocharunchitt et al., 2014).

For further investigation, the KEGG database was used and the expressed proteins were identified in four categories: "metabolism," "genetic information processing," "environmental information processing," and "cellular processes" (**Figure 3**). It displayed that "Metabolism" with seven subcategories was the most enriched, which verified the active metabolic changes after a cold response. Among these subcategories, more proteins were enriched in metabolic related pathways: carbohydrate metabolism, nucleotide metabolism, amino acid biosynthesis, and translation. Similar pathways involved in cold response were also described in *L. monocytogenes* and *E. coli* (Cacace et al., 2010; Kocharunchitt et al., 2014).

Analysis of Differentially Expressed Proteins at Low Temperature

To investigate the alteration of metabolism related to growth profile under cold temperature over time, differentially expressed proteins were investigated at different time points in two isolates, 44B and II7D (with low and high growth ability at 4°C). Differentially expressed proteins of 44B and II7D under cold stress for 2 h (early stage, T1) and 24 h (late stage, T2) were compared (**Figure 4**).

Differentially expressed proteins were classified into 20 COGs functional groups with a relative fold change [$\log_2$ (FC) > 1.2 and $\log_2$ (FC) < -0.8, p < 0.05]. The expressed protein response to the early stage of the cold response (T1) was enriched into 17 functional clusters. Of these, the most predominant categories were "amino acid transport and metabolism," "translation, ribosomal structure, and biogenesis," "carbohydrate transport and metabolism," "cell motility," and also

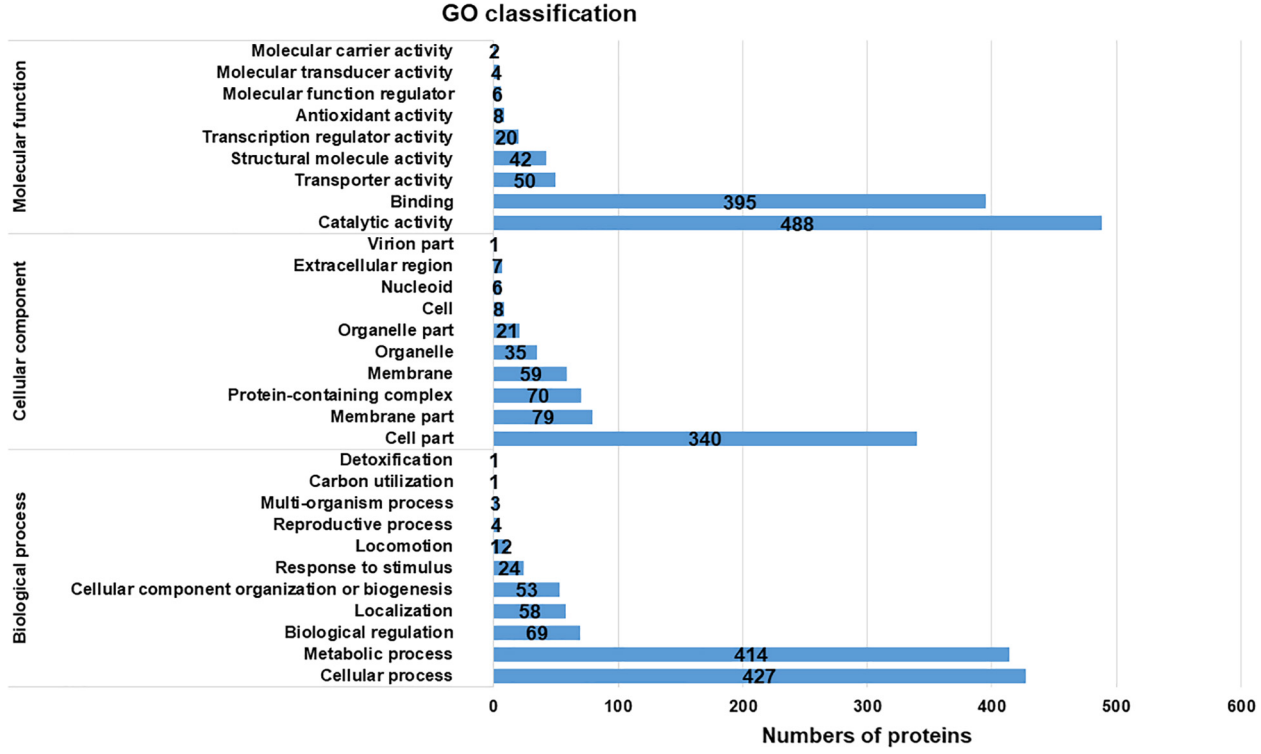

FIGURE 2 | Gene Ontology classification of the total assembled uniproteins.

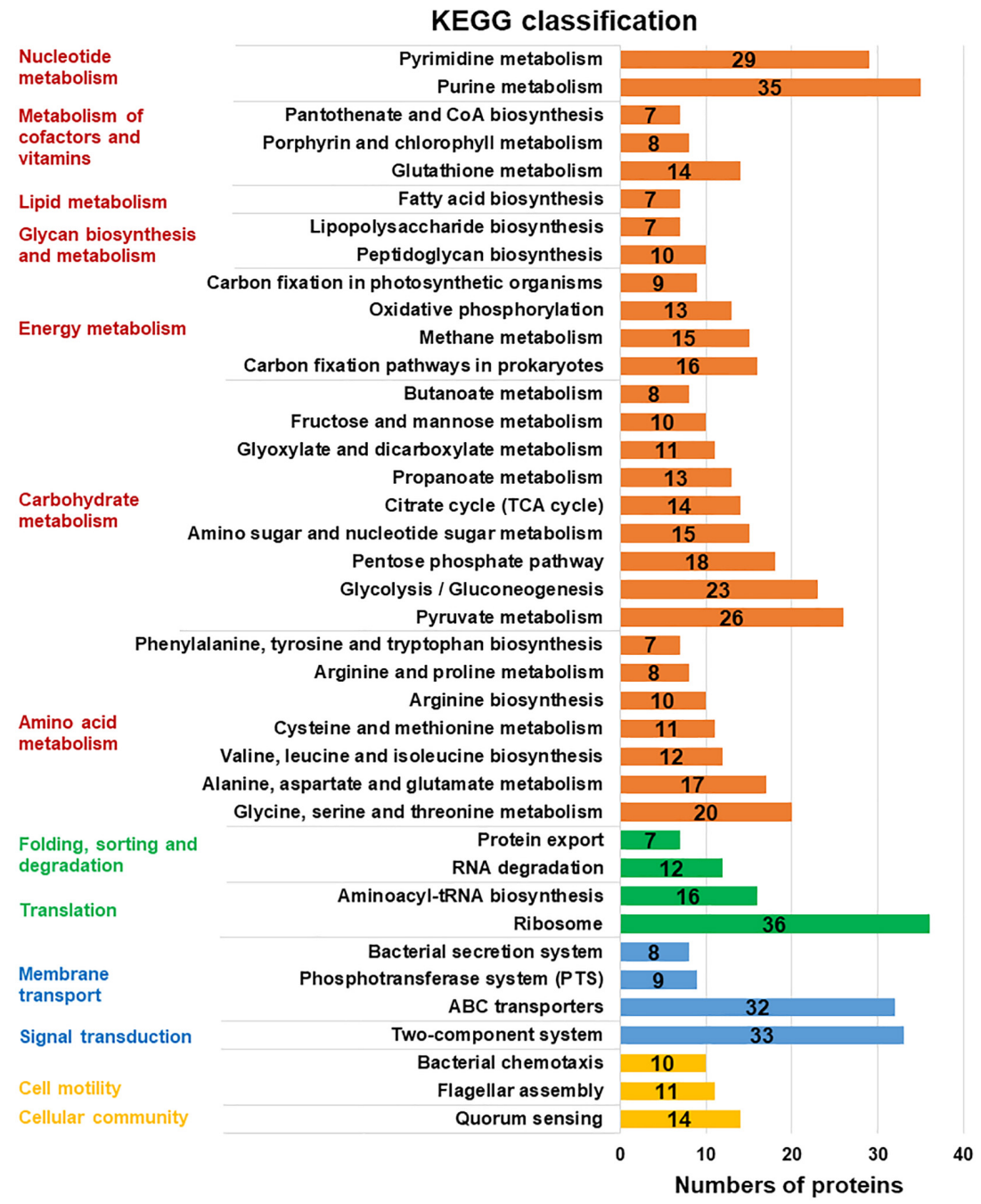

FIGURE 3 | KEGG pathway clusters of assembled uniproteins. Metabolic pathways in different functional groups involved in cold response were classified with KEGG database in four related categories (protein numbers of each group higher than 10): "metabolism" in red, "genetic information processing" in green, "environmental information processing" in blue, and the "cellular processes" in yellow. The subcategory titles were also represented.

"transcription." For the late stage of the cold response (T2), a high abundance of proteins was observed for categories of "amino acid transport and metabolism," "translation, ribosomal structure, and biogenesis," "cell wall/membrane/envelope biogenesis," and "carbohydrate transport and metabolism." In addition, a high abundance of the proteins belonging to "general function prediction only" was also found in both stages (**Figures 4A,B**). Throughout the whole testing course (T1–T2), proteins in specific functions of amino acid transport and metabolism (E), translation, ribosomal structure and biogenesis (J), carbohydrate transport and metabolism (G), and energy production and conversion (C) had higher enrichment in both T1 and T2. Hence, the proteins are involved mostly in metabolism in response to cold. Similarly, the

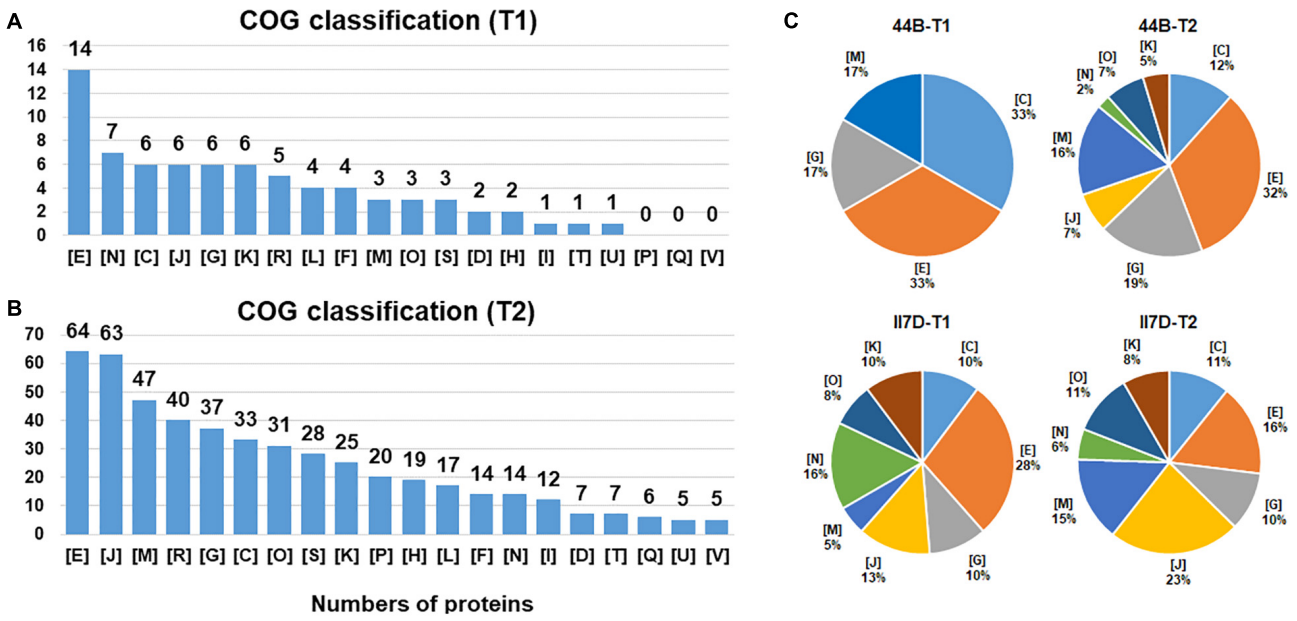

FIGURE 4 | Functional category distribution of proteins identified from *Y. enterocolitica* cells at low temperature. **(A)** The number of identified proteins belong to each functional category is shown under cold stress after 2 h (T1). **(B)** The number of identified proteins belong to each functional category is shown under cold stress after 24 h (T2). **(C)** The percentage of differential expressed proteins that belong to each functional category in II7D and 44B is shown under cold stress in two time (T1 and T2). COG categories are as follows: C: energy production and conversion; D: cell division and chromosome partitioning; E: amino acid transport and metabolism; F: nucleotide transport and metabolism; G: carbohydrate transport and metabolism; H: coenzyme metabolism; I: lipid metabolism; J: translation, ribosomal structure and biogenesis, K: transcription; L: DNA replication, recombination and repair; M: cell wall/membrane/envelope biogenesis; N: cell motility; O: post translational modification, protein turnover, chaperones; P: inorganic ion transport and metabolism; Q: secondary metabolites biosynthesis, transport and catabolism; R: general function prediction only; S: function unknown; T: signal transduction mechanisms; U: intracellular trafficking, secretion, and vesicular transport; V: defense mechanisms.

high abundances of proteins regarding metabolism-related pathways and metabolic process were also investigated in the KEGG and GO analysis. Hence, we assume that the major cold-responsive proteins participate in the metabolic regulation of cells.

However, differences in protein abundance were observed between T1 and T2. Especially, protein abundance existed mostly in the clusters of cell motility (N) and transcription (K) in T1 while cell wall/membrane/envelope biogenesis (M) and post-translational modification, protein turnover, chaperones (O) in T2. This result indicates that the effects of the cold response on protein levels differ in the early and late stages. The time-dependent differences in protein categories were also found in *E. coli* in response to temperature and water-activity changes and were closely related to the cultivability after the temperature downshift (King et al., 2016). Different phases including adaptation and re-growth phases could be divided based on clustering analyses. Additionally, various protein categories were involved such as energy metabolism, DNA repair system, amino acid biosynthetic pathways, and carbohydrate catabolism (King et al., 2016).

According to the COG classification, eight protein clusters with the most protein abundance in T1 or T2 were chosen to compare the differences between strain 44B and II7D (**Figure 4C**). Compared with the protein abundance in the other three pie charts, protein clusters of (K), (O), (J), and (N) were undetectable in the early stage of 44B (T1) and the proportions of these proteins in all selected proteins in 44B (T2) were lower than those in II7D (T1) and II7D (T2). This result demonstrates that the biogenesis of responding proteins in 44B lags behind II7D under cold stress. Meanwhile, the proteins in clusters of (K), (O), and (J) represent key processes of protein biosynthesis. Hence, lower abundances of these proteins in 44B (T1) and 44B (T2) suggested that synthesis of general proteins might be inhibited in 44B compared to strain II7D. As was shown in many bacteria (e.g., *E. coli*), the arrest of cell growth upon temperature downshift is caused by the severe inhibition of general protein synthesis (Phadtare, 2004). The inhibition of general proteins in 44B (both in T1 and T2) might be the reason for low growth ability at low temperature. Considering the expressional repression of cold acclimation genes in 44B (**Figure 1B**), the inhibition might be involved in synthesis of cold acclimation proteins, which are essential for cold adaptation during prolonged growth.

In addition, a lower abundance of protein cluster (N) related to the "cell motility" was also mentioned in 44B. Base on the proteomic results, some cold-responsive proteins related to flagella and chemotaxis were detected in II7D but not in 44B (data not shown). For example, the Flg family, used for flagellar assembly and motility, are temperature-dependent in *E. coli* and other bacteria (Phadtare, 2012;

Osterman et al., 2015). The chemotaxis protein, Che family is essential for motility and cold response (Burkart et al., 1998; Liu et al., 2014). According to the transcriptional analysis in **Figure 1B**, the correlation between motility and growth ability was demonstrated due to the different expressional changes of motility-related genes *fleC* (homologous to *fliC* and encoding Flagellin), *fliS* (putative cytoplasmic chaperone), and YE2848 (putative chemotaxis methyl-accepting transducer) in three isolates. Meanwhile, the Flagellin was detectable only in II7D but not in 44B in proteomic analysis (other related genes were not found). Since it is critical in motility and cold response in *Salmonella enterica* (Elhadad et al., 2015; Michaux et al., 2017), the involvement of motility in cold response might be confirmed.

On the other hand, the percentages of clusters of energy production and conversion (C), carbohydrate transport and metabolism (G), and cell wall/membrane/envelope biogenesis (M) in 44B (T1) are higher than those in 44B (T2), II7D (T1), and II7D (T2). Considering the high abundance of proteins related to carbohydrate metabolism and cell wall/membrane/envelope biogenesis, but low enrichment of proteins related to functional protein synthesis in strain 44B (T1), we might assume that 44B uses a high rate of energy for the cell wall structure, instead of initial growth at cold response. As an important protective structure against adverse environmental conditions, the cell membrane plays an important role in stress response. Previously, it was extensively discussed that membrane lipopolysaccharide, cell membrane, and the membrane fluidity contribute to temperature adaptation in bacteria (Carty et al., 1999; Phadtare, 2004; Storz and Hengge, 2010).

Motility at Low Temperature

To investigate the physiological changes in cold response, motility assays at low temperatures were performed on three isolates (44B, 8081, and II7D) with different growth profiles at low temperatures. All three strains showed motility at 28°C; however, at the temperature of 4°C, only II7D was motile (**Figure 5**).

As shown in our transcriptional analysis, the expression of motility-related genes (*fliS* and *fleC*) increased under cold stress at 4°C and their expression was increased earlier in strain 44B than in the other two strains (**Figure 1B**). Meanwhile, a lower abundance of proteins was present in the "cell motility" group in 44B, which was consistent with the lower growth ability in 44B than II7D. Moreover, the differential growth ability correlates with motility in the three strains (only the strain with high growth ability was motile) at low temperature. Based on the results from transcriptional and proteomic analysis, the different induction of the motility-related genes and proteins among isolates with different growth behaviors indicated the close link between cell motility and growth ability, which has been described previously in *Y. enterocolitica* (Kapatral et al., 1996). However, due to the wide range of factors with complex mechanisms in regulating motility, how cell motility was affected by or contributed to the growth ability after cold response remains unclear (Young et al., 1999; Mukherjee et al., 2013; Xu et al., 2014).

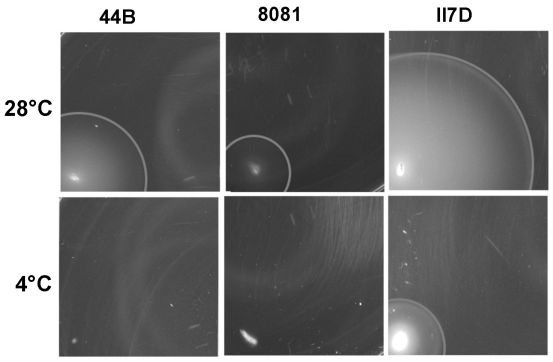

FIGURE 5 | Motility of *Y. enterocolitica* strains at low temperature. Motility was tested on motility agar plate. Single colonies were stabbed on to motility agar plates and incubated initially for 2 h at 37°C to start the assay with non-motile bacteria. The plates were subsequently incubated at 28°C (for 21 h) and 4°C (for 44 h). The motility was assessed by measuring the diameters of migration zone of strains. Values of the migration zone diameters (means ± SEM) in each strains were tested of six independent experiments: 24.92 ± 0.239 cm (44B at 28°C), 11.58 ± 0.201 cm (8081 at 28°C), 49.00 ± 0.966 cm (II7D at 28°C), and 17.83 ± 0.105 cm (II7D at 4°C). No migration zone was detectable in 44B or 8081 at 4°C.

Cell Membrane and Fluidity at Low Temperature

To test other factors corresponding to membrane activity in cold response, fluidity assays were performed at 4°C on three isolates (44B, 8081, and II7D). All three tested strains showed stable fluidity under the temperature of 28°C in 48 h, while the membrane fluidity of 44B increased significantly at 4°C at 2 h and decreased to the normal level at 4°C after 24 h (**Figure 6A**). According to the results of fluidity, the membrane fluidity maintained at the normal level in both strains 8081 and II7D, but not in 44B. These results indicated that the balance of the membrane fluidity was changed in response to cold stress in 44B at 2 h. This finding might be correlated to the high protein abundance in the functional cluster of cell wall/membrane/envelope biogenesis in 44B at T1. Therefore, the differences in growth abilities at low temperature might be related to the maintenance of cell fluidity. The similar roles of membrane fluidity have been demonstrated in cold and other stresses in many bacteria (Yoon et al., 2015; Eberlein et al., 2018). However, the fluidities are regulated by various mechanisms in different bacteria; e.g., *E. coli* (Carty et al., 1999), *B. subtilis* (Aguilar et al., 2001), and *Salmonella* (Wollenweber et al., 1983; Ricke et al., 2018).

To investigate the regulatory factors of membrane fluidity in *Y. enterocolitica*, groups of genes regarding outer membrane proteins and lipid A biosynthesis were selected according to previous studies (Dekker, 2000; Nikaido, 2003; Barria et al., 2013; Hussain and Bernstein, 2018; Robinson, 2019). Transcriptional changes in these genes were investigated under cold stress for 2 h in three isolates with the primers listed in **Supplementary Table S3**. In strain 44B, significantly higher expression of *yaeT*, *yfgL*, *dapX*, and *pldA* and lower expression of *yfiO* and *lpxP* was observed compared to the other isolates (**Figure 6B**). The

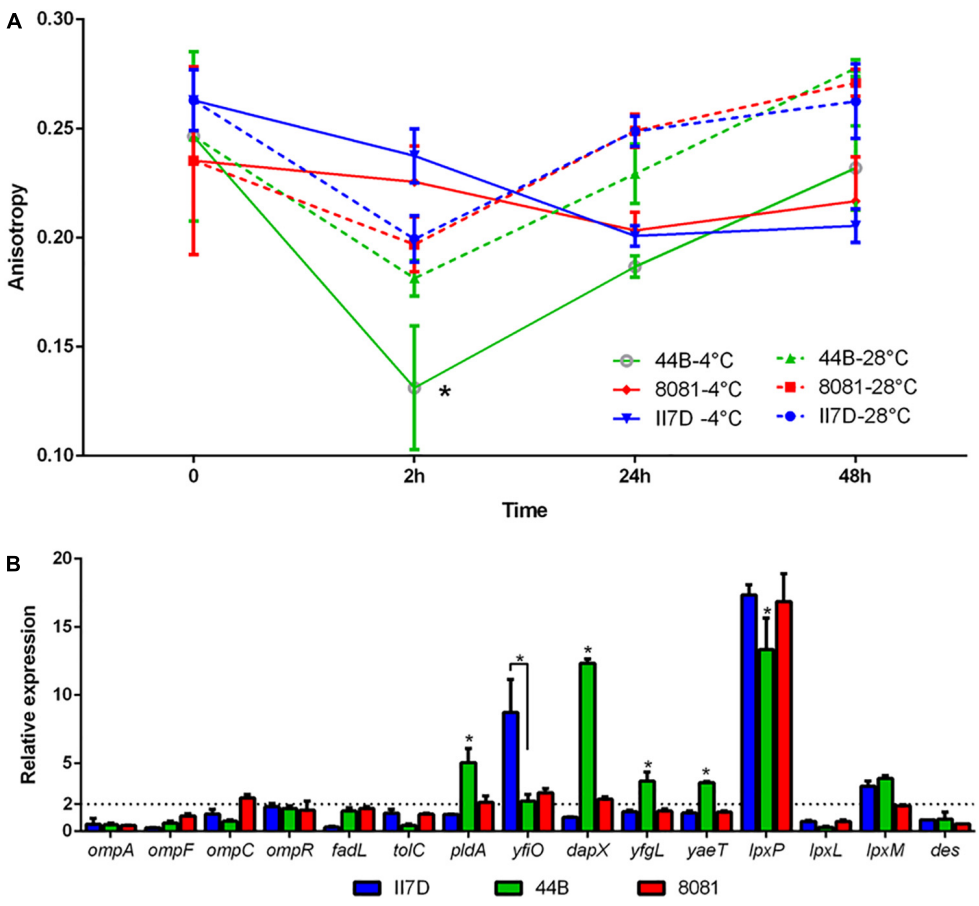

FIGURE 6 | Membrane fluidity and transcriptional changes of membrane-related genes in *Y. enterocolitica* strains at low temperature. **(A)** Fluidity assays in II7D, 44B, and 8081 under cold stress for 2, 24, and 48 h. Anisotropy value represented the membrane fluidity (higher anisotropy means lower fluidity). **(B)** Expressional changes of the membrane related genes were detected using RT-qPCR and were normalized to the reference gene *polA*. Specific values of relative gene expression are shown as the means ± SEM of four independent experiments. The line parallel to the *x*-axis represents a biologically relevant induction at 2 (fold-change). Statistically significant difference compared with the control according to multiple comparisons (*$p < 0.05$).

four outer membrane protein assembly factors (encoded by *yaeT*, *yfgL*, *dapX*, and *yfiO*) were found in proteomic analysis, in which, BamC encoded by *dapX* was upregulated significantly in 44B. The differential expression of these genes and proteins might be involved in fluidity regulation in response to cold. Similar functions of the outer membrane protein YaeT, DapX, and YfgL (homologous to insert β-barrel proteins in *E. coli*) were shown in previous research in response to cold (Macintyre and Henning, 1990; Onufryk et al., 2005; Wu et al., 2005; Begic and Worobec, 2006; Sklar et al., 2007; Rollauer et al., 2015). Outer membrane phospholipase A (encoded by *pldA*), which is activated under various stress conditions, presents in the outer membrane of Gram-negative bacteria. Its possible role is maintaining the cell envelope integrity and permeabilization, which is related to temperature (Dekker, 2000; Belosludtsev et al., 2014). The different expressions of *pldA* gene and protein among isolates suggested the possible involvement of outer membrane phospholipase A in fluidity maintenance under cold stress. However, their functions in cold response still remain to be elucidated in *Y. enterocolitica*.

Des and LpxP (encoded by *des* and *lpxP* genes) are two fluidity-generated enzymes in *B. subtilis* and *E. coli*. In *B. subtilis*, upon a drop in temperature, the Des protein is synthesized and desaturates the acyl chains of membrane phospholipids to increase the membrane fluidity (Aguilar et al., 2001; Albanesi et al., 2004). Furthermore, in *E. coli*, cold-induced acyltransferase LpxP helps to attach more unsaturated fatty acids (palmitoleate instead of laurate attached at normal temperature by LpxL) to lipid A, thus increasing membrane fluidity and lowering its phase transition temperature, counteracting the effect of low temperature (Vorachek-Warren et al., 2002). In our study, no significant difference in the expression of *des* gene was observed among the three isolates and the Des protein did not induced, which implied that the regulation of Des in *Y. enterocolitica* might not be as important as that in *B. subtilis*. Meanwhile, expression of the cold induced gene *lpxP* was significantly lower in 44B and the related protein were induced differently between II7D and 44B according to proteomic analysis (**Supplementary Table S4**). Therefore, membrane fluidity related to growth ability in *Y. enterocolitica*

might be regulated by LpxP in cold adaptation, which is identical to *E. coli*.

Proteomic Overview of the Cold Response in *Y. enterocolitica*

Cold response involved a series of complex and significant changes in the abundance of proteins in many processes and pathways rather than a simple increase or decrease in a specific category. To present an overview of the cold response of *Y. enterocolitica*, the upregulated proteins under cold stress were selected according to the main COG functional categories mentioned in this study. These particular proteins probably represented the key determinants that allow life at low temperature. Top KEGG pathways (including the BRITE hierarchies) were selected according to the proteins in COG categories (listed in **Supplementary Table S4**). Proteomic overview and the predicted regulation in cold response are presented in **Figure 7**.

First, a high abundance of proteins was observed associated with protein biosynthetic processes, such as transcriptional, translational, and ribosomal, and post-translational processes (related COG categories are shown in yellow boxes). These proteins were involved predominate in transcription factors, RNA degradation, peptidases and inhibitors, ribosome biogenesis, and aminoacyl-tRNA biosynthesis. Numbers of proteins related ribosome biogenesis [such as the transcription termination/anti-termination protein NusA, ribosome-associated inhibitor A (encoded by *raiA*), and ribosomal RNA small subunit methyltransferase B (encoded by *rsmB*)] were induced in response to cold. These related protein associated with cold stress was also reported in *E. coli* and other bacteria (Burakovsky et al., 2012; Di Pietro et al., 2013). Meanwhile, functions of the proteins induced in this study

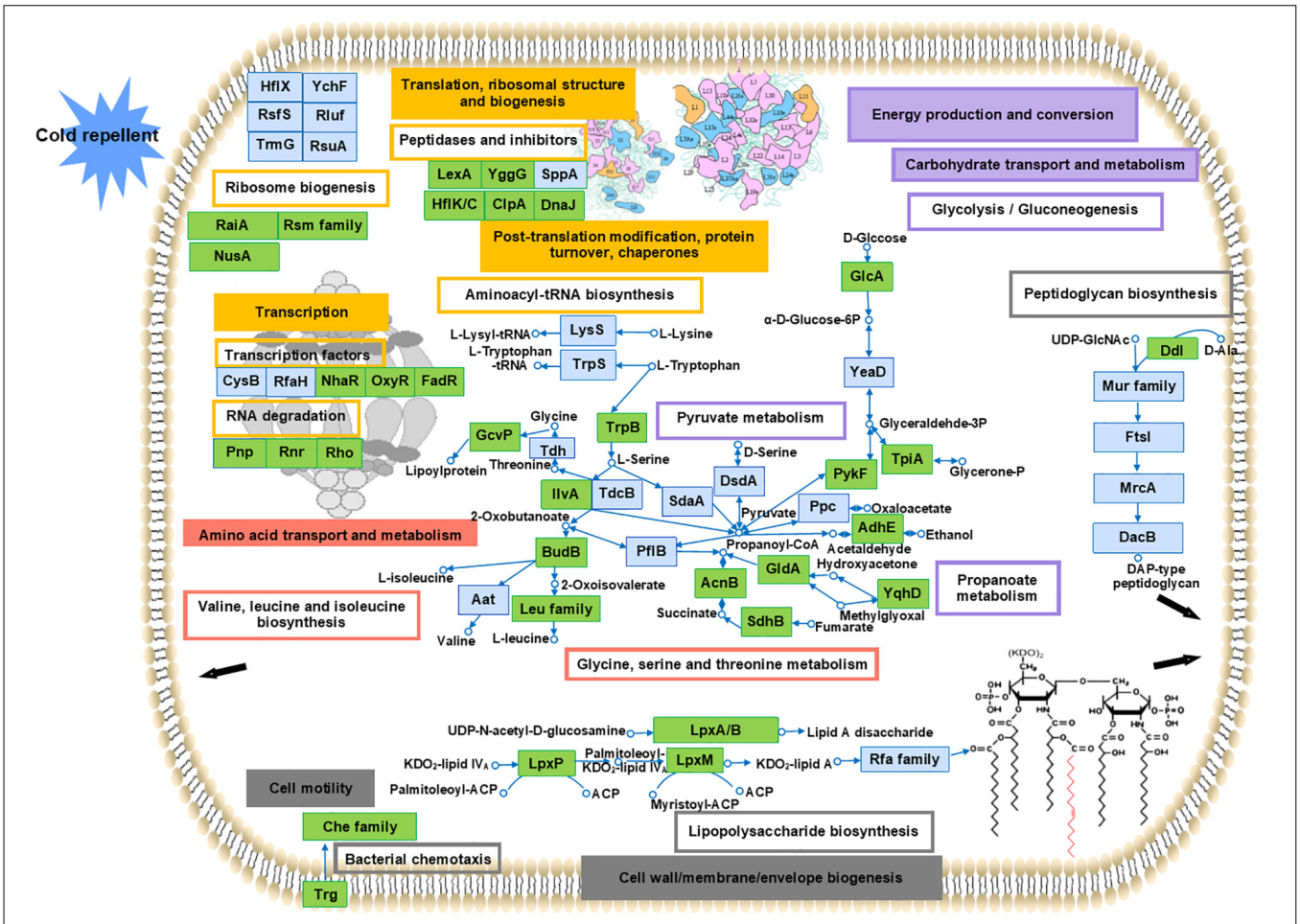

FIGURE 7 | Overview of the cold response of *Y. enterocolitica* at proteomic level. Proteins were selected in the main COG functional categories and distinguished. Boxes with colored border represented the related pathways (including KEGG BRITE hierarchies). The main KEGG pathways reported in each category were presented: transcription factors, RNA degradation, ribosome biogenesis, peptidases and inhibitor, and aminoacyl-tRNA biosynthesis (in yellow); glycine, serine, and threonine metabolism and valine, leucine, and isoleucine biosynthesis (in red); lipopolysaccharide biosynthesis, peptidoglycan biosynthesis, and bacterial chemotaxis (in gray), glycolysis/gluconeogenesis, pyruvate metabolism, and propanoate metabolism (in purple). The related COG categories were marked with the same colors in boxes accordingly. The proteins were displayed in blue boxes representing the involvement in the related functional categories and pathways in this study. The green boxes were used for the proteins also clarified in cold response in other researches. Detailed information is listed in **Supplementary Table S4**.

like ribosomal silencing factor RsfS, GTPase HflX, and ribosome-binding ATPase YchF under cold stress have not been clarified previously. Since some of them are involved in other stress response like heat, oxidative, and nutrient stress (Starosta et al., 2014; Hannemann et al., 2016; Dey et al., 2018), their potential roles under cold stress should be investigated. The proteins associated with peptidases and inhibitor such as LexA repressor, lipoprotein (encoded by *yggG*), and Protein HflK were induced under cold stress in this study. The cold-responsive function of these proteins was also found previously in other research (Phadtare and Inouye, 2004; Burakovsky et al., 2012; Jian et al., 2015). Besides, some transcription factors (encoded by *nhaR*, *oxyR*, *fadR*, *cysB*, and *rfaH*) were also involved in regulation of cold response. Since the essential cold-responsive roles of these transcription factors (encoded by *nhaR*, *oxyR*, and *fadR)* were investigated in *E. coli* (White-Ziegler et al., 2008), *Vibrio vulnificus* (Limthammahisorn et al., 2008), and *Moraxella catarrhalis* (Spaniol et al., 2013), transcription factors should also be focused on in *Y. enterocolitica* cold response.

A number of proteins involved in specific amino acids biosynthesis may reflect their importance in mediating survival under cold stress. In our research, the induced proteins participated in biosynthesis of various amino acids under cold stress (related COG categories shown in red boxes). These proteins are associated with the biosynthesis and metabolism of glycine, serine, and threonine (encoded by *trpB*, *gcvP*, *ilvA*, *tdh*, etc.), and valine, leucine, and isoleucine (encoded by *leuA*, *leuB*, *leuC*, *leuD*, *budB*, etc.). Similar amino acids have been demonstrated in previous studies to aid tolerance under cold stress conditions in many other bacteria (Fonseca et al., 2011; King et al., 2016).

Second, proteins associated with cell membrane and motility were identified (related COG categories shown in gray boxes). Certain proteins were identified in pathways, such as lipopolysaccharide biosynthesis (encoded by *lpxA/B/P/M* and *rfaC/Q*), peptidoglycan biosynthesis (encoded by *murC/E*, *ddl*, *dacB*, etc.), and bacterial chemotaxis (encoded by *trg* and *cheB/D/Z*). The cold-responsive functions have been reported previously in many proteins mentioned in **Supplementary Table S4**. However, although the genes related to motility (*fliS* and YE2848) were detected in our transcriptional, the induction of them cannot be detected in our proteomic results. According to the proteomic data, almost all the proteins related to flagellar assembly were downregulated. The cold-responsive effect of flagella on cell motility at the protein level is unknown.

Meanwhile, valine, leucine, and isoleucine, as the branched-chain amino acids and the precursors for biosynthesis of iso- and anteiso-branched-chain fatty acids, were utilized to regulate the membrane fluidity in response to cold in certain bacteria (Grau and de Mendoza, 1993; Annous et al., 1997; Klein et al., 1999). Levels of isoleucine and leucine significantly increase under cold stress in *E. coli* (Jozefczuk et al., 2010), and the expression of related genes (*leuA/B/C/D* and *ilvB/C/D/E/H*) was also elevated in *Thermoanaerobacter tengcongensis* (Liu et al., 2014). Based on our proteomic results, the induction of l*euA/B/C/D* encoding proteins was only detected in 44B, which implied the indispensable regulation of these branched-chain amino acids. However, the growth ability under cold stress of 44B was detected worse than II7D, which suggested that multiple pathways related to motility might be applied in cold response.

The considerable involvement of proteins has been detected and the transcriptional and physiological investigation associated with motility and fluidity contributes to our understanding of cold-response regulation of motility and membrane fluidity.

Additionally, certain pathways in energy production and conversion and carbohydrate transport and metabolism were also involved in this study (listed in **Supplementary Table S4**). The complex processes and pathways in cold response of *Y. enterocolitica* and the specific functions of other individual proteins predicted in the proteomic results are required to be investigated during cold adaptation.

This study demonstrates the strain-specific cold response of *Y. enterocolitica* at 4°C, which is time-dependent, including cold acclimation and adaptation. The transcriptional analysis revealed the importance of the induction and repression of cold-shock genes in cold acclimation as well as the resumption of the non-cold shock genes in prolonged cold adaptation. Meanwhile, the time-dependent response at protein level was also found and the cold-responsive proteins identified in proteomic analysis were closely related to protein synthesis, cell membrane parts and cell motility. Additionally, the physiological processes in cell fluidity and motility might be responsible for differential growth abilities at low temperatures. By combining different approaches, cold response was described systematically, providing a better understanding of the significant physiological processes involved in cold stress of *Y. enterocolitica.*

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article and **Supplementary Material**.

AUTHOR CONTRIBUTIONS

CL contributed to designing, carrying out the experiment, and writing the manuscript. JM provided assistance for the proteome experiment and reviewed the manuscript. CT provided assistance for the experiment. TA reviewed the manuscript and gave advice. CR reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.03037/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

CHAPTER 5 DISCUSSION

5.1 Prevalence and the predicted transmission routes of *Y. enterocolitica* in seafood

Y. enterocolitica is ubiquitous in nature and routinely isolated from a broad range of environment, animals and food products. In this study, the prevalence of *Y. enterocolitica* in seafood samples from retail markets in Berlin (Germany) was investigated. The total prevalence in seafood samples was 2.7% (6 of 220 samples). A similar prevalence was also found in other studies, e.g. 1% prevalence of *Y. enterocolitica* in frozen crustaceans or mollusks samples in Italy [192], 2.7% (1/37) in shrimp sample in Thailand [193] and oysters, mussels and other seafood [194, 195] [196]. However, some studies found higher prevalences in fresh seafood samples: e,g, in shrimps (13%) and blue crabs (21% prevalence) in the US and raw seafood in Malaysia (45.5% prevalence) [197, 198]. Differences might be explained by different detecting methods and sampling areas. In our study, 220 samples were collected and multiple detecting methods were used and combined.

In this study, all *Y. enterocolitica* isolates belong to biotype 1A, which lacks the pYV virulence plasmid. However, the pathogenic potential of 1A isolates is still under debate [121, 122]. Although most chromosomal virulence genes occurred in the other *Y. enterocolitica* biotypes, biotype 1A strains have been repeatedly isolated from patients with gastrointestinal symptoms [123]. Moreover, at least two outbreaks of Yersiniosis have been reported to be caused by biotype 1A [199, 200]. Combining that information, although no highly virulent strains were detected in seafood, seafood may -nonetheless- pose a risk for consumer's health.
Although the transmission routes of seafood are still poorly understood, the assumption for the presence of *Y. enterocolitica* in seafood within the food chain can be proposed. Many opportunities for *Yersinia* to enter food from aquatic habitats, which present the possibility of the contamination in seafood. For example, the presence of *Y. enterocolitica* in soil, freshwater ecosystems and birds have been provided previously. In addition, contamination by human or animal feces and post-harvest handling and processing may also pose a risk of seafood contamination [201].

5.2 Network of cold shock response

5.2.1 Cold acclimation and long-term adaptation in *Y. enterocolitica*

Usually, gene expression induction has been studied immediately after sudden environmental changes, e.g., cold shock, acid shock or heat shock while much less effort has been dedicated to analyzing gene expression during prolonged growth under a specific environmental stress. Our work focused not only on the short term of the cold response but also further investigated the long-term cold adaptation. The transcriptional and translational analysis at low temperature were investigated in related genes and proteins with a wide range of functions. Among these, cold-shock proteins, PNPase, non-cold-shock proteins, and other complex metabolic processes were involved.

5.2.1.1 Cold-shock proteins

According to the sequencing and annotation of *Y. enterocolitica* genomes, *cspA* and *cspB* are the most studied and strongly expressed genes during the cold response [174, 175, 202]. In this research, the gene expression of *cspA* and *cspB* increased up to thousand fold after temperature down shift and subsequently decreased under cold

stress at around 4 h. This result confirmed the essential role of cold-shock proteins in *Y. enterocolitica* under cold stress. Although their general characteristic as cold-response proteins was confirmed, further studies should be carried out on the function of these proteins. In addition, in isolates with low growth abilities at low temperatures, the expression of *cspB* genes decreased slowly and the relative expression was higher than in other isolates (with higher growth abilities) after cold stress. Hence, different transcriptional levels of *cspB* in isolates might be associated with specific growth abilities at low temperatures. Similarly, from a previous research, CspB is reported be able to protect cells from damage caused by low temperatures and it is required for cell viability at low temperatures in e.g. *B. subtilis*, *S. aureus* and *S. cerevisiae* [203-205]. However, the role of *cspB* in cold response has not been clarified yet. According to a previous study, under cold stress, the stabilization of secondary structures of RNA and DNA was firstly affected and it makes difficulties in translation, transcription and replication [175, 206, 207]. Cold-shock proteins are involved in activating transcription and unwinding or masking RNA molecules [208-210]. It was shown that *cspA* and *cspB* act as a transcriptional activator and a chaperone of both DNA and RNA. It can facilitate proper transcription and replication of DNA and help translation by preventing the formation of stable secondary structures in mRNAs under cold stress in *E. coli*, *Pasteurella multocida*, *Burkholderia thailandensis* and *B. subtilis* [211-214]. Some Csps were also reported to be involved in various cellular processes to promote normal growth and stress adaptation responses [142]. Therefore, Csps seem to have a wider role in stress tolerance of bacteria than previously assumed. In *B. subtilis*, the *csp* gene is essential for viability and needed during non-shock growth [215]. During NaCl, pH, and ethanol stress response in *C. botulinum*, the *cspA* and *cspC* also affect motility and flagella formation [216]. Enteropathogenic *Yersinia* encode several *csp*s which show a high homology to those of *E. coli*. However, it is not known what the role of other *csp* genes in stress response of enteropathogenic *Yersinia* is.

In addition, two other genes (*gltP* and *uhpC*) were also induced at low temperature in this study. The expression of these two genes increased rapidly after a cold stimulation and then decreased over time (similar to the *csp* gene). Glutamate-aspartate symport protein (encoding by *gltP*) is an amino acid transport protein involved in the accumulation of stabilizing organic compounds under different stress conditions [217, 218]. Recently, the effect of aspartic acid and glutamate on metabolism and acid stress resistance of *Acetobacter pasteurianus* has been reported. It shows that aspartic acid and glutamate have an essential impact on stress response [219]. In *L. monocytogenes* and *B. subtilis*, the role of glycine betaine were also characterized in cold-protective effects [218, 220]. In this study, *gltP* showed great influence on cold-acclimation in *Y. enterocolitica*. Similar studies have been carried out on the amino transporter system especially glutamate transport system in *E. coli* and some other bacteria like *Pyrococcus horikoshii* and *Rhodobacter sphaeroides* [221-223]. However, it is still unknown how *gltP* participates in amino acid transport system regulating cold response in low temperature in *Y. enterocolitica*. *Uhp*C as sensing regulation gene, encodes a kind of hexose phosphate transport system regulatory protein [224]. In our research and others, *uhp*C was induced significantly during cold shock. The function of *uhp*C in cold adaptation may depend on this hexose phosphate transport system regulatory protein. In the Uhp system of *E. coli*, signaling is initiated through sensing of extracellular glucose 6-phosphate by membrane-bound UhpC, which in turn modulates the histidine-protein kinase UhpB. Together with the cytoplasmic response regulator UhpA, they constitute a typical two-component regulatory system based on His-to-Asp phosphoryl transfer [225].

In this study, *uhp*C was mentioned but no further clarification has been made after cold stimulation.

5.2.1.2 Polynucleotide phosphorylase (PNPase)

Before bacterial cells start to resume growth after cold shock, other proteins need to be produced. PNPase, encoded by *pnp* gene, is involved in RNA decay, which is required for the production of these proteins. In our study, the expression of *pnp* gene was induced, which indicated that *pnp* is required for the cold response in *Y. enterocolitica*. During a cold response, different induction of *pnp* expression was found in specific isolates with various growth ability. This results verified the essentiality of the *pnp* gene in cold adaptation and the continuous higher expression of *pnp* gene implies the higher demand of PNPase and *pnp* in isolates with low growth ability at low temperatures. According to a previous study, the indispensable role of *pnp* in the cold response of *Y. enterocolitica* has already been demonstrated [226]. It was reported, that *pnp* has an upstream promotor region with the ATTGG temperature dependent motif in *Y. enterocolitcia*, which regulates gene expression by selectively degrading Csp mRNAs and thereby to enable growth to resume at low temperature [149, 227] [177].

Many other studies have been done to indicate the function of PNP verifying its regulation role in cold response. The same function of PNPase was also found in many bacteria including: *E. coli* [228], *Salmonella enterica* [229], *Campylobacter jejuni* [230] and *B. subtilis* [231]. Combining this information, the expression of *pnp* is temperature-dependent and the cold shock leads to an increase in PNPase levels [232-234] [177]. So that it might explain the result in our study that *pnp* gene expression increased after temperature down-shift but in different levels among isolates: the repression of Csp generation might be not accomplished in isolate with lower growth ability at low temperatures. In addition, as it was reported previously, after the repression of Csp production, the growth reinitiated at the end of the acclimation phase [235]. Therefore, the RNA degradation of Csps by PNPase was indispensable for cold acclimation and growth resumption. PNPase is also reported of having other functions in stress response in many bacteria. In *E. coli*, PNPase and RNase II are the major 3'-exonucleases involved in RNA degradation and are involved in biofilm formation [236-238]. Furthermore, the activity of PNPase is modulated by a number of small molecule effectors including ATP, cyclic di-GMP, citrate and some other metabolites [239-242].

5.2.1.3 Non-cold-shock proteins

The induction of cold-shock proteins (Csps) exists in all psychrotrophs, mesophiles and thermophiles [145, 243]. Meanwhile, in psychrotrophic bacteria such as *Arthrobactor globiformis* and *Pseudomonas fragi*, some cold-response proteins are synthesized at relatively moderate levels and the induction of these proteins can prolong in response to continuous growth at low temperatures [244, 245]. These proteins are of particular importance since they differentiate psychrotrophs from mesophiles, and this group of non-cold-shock proteins is probably one of the key determinants that allow bacterial survival at low temperature [146]. As it was reported previously, after the repression of Csp production, the growth reinitiated at the end of the acclimation phase [235]. In our study, differences in cold response were detected on transcriptional levels by genes YE1436, *fleC*, *fliS* and YE2848. According to the expressional investigation, slighter increase in expression of these genes was detected in the isolate with low growth ability at low temperature. Regarding the growth ability, the isolate with low growth ability might not be able to produce several non-cold shock proteins and allow growth during

prolonged low temperatures in cold adaptation [145, 146, 245, 246]. After the cold acclimation, the expression of non-cold shock genes has not been resumed in this isolate, arresting the transition from acclimation to cell growth.

Notably, the regulation of these non-cold-shock proteins and other general proteins at proteomic level were also investigated in our research. A high abundance of proteins was observed associated with protein biosynthetic processes based on the COG and KEGG annotation. These proteins are related to transcriptional, translational and ribosomal, and post-translational processes. These proteins were involved in transcription factors, RNA degradation, peptidases and inhibitors, ribosome biogenesis and aminoacyl-tRNA biosynthesis. For example, transcription termination/anti-termination protein NusA, ribosome-associated inhibitor A (encoded by *raiA*) and ribosomal RNA small subunit methyltransferase B (encoded by *rsmB*) were induced in response. These proteins associated with cold stress were also reported in *E. coli* and other bacteria [247, 248]. The proteins associated with peptidases and inhibitors such as LexA repressor, lipoprotein (encoded by *yggG*) and protein HflK were induced under cold stress in this study. The cold-response function of these proteins was also found previously in other studies [247, 249, 250]. Besides, some transcription factors (encoded by *nhaR*, *oxyR*, *fadR*, *cysB* and *rfaH*) were also involved in regulation of cold response and similar cold-response roles of these transcription factors were investigated in *E. coli* [251], *Vibrio vulnificus* [252] and *Moraxella catarrhalis* [253]. All these reflected the need for protein biosynthesis under cold stress. Previously, proteomic studies have compared the relative abundance of protein synthesized in *P. haloplanktis*, 30 % of the upregulated proteins at 4°C were found to be directly related to protein synthesis and it was concluded that protein synthesis may be a limiting step for growth in the cold [254]. The proteomic response of the psychrophilic *Colwellia psychrerythraea* at -10 °C resulted in an increase in the abundance of translation processes and protein synthesis [255].

Consequently, the cold response of *Y. enterocolitica* includes both, cold acclimation and prolonged adaptation. The isolate-specific ability to survive under cold stress depends on the capacity of enabling transient induction and effective repression of cold-shock gene in cold acclimation. Meanwhile, the resumption of the non-cold shock gene expression was also required in prolonged cold adaptation. So the pronounced separation between cold response and long-term adaptation may be a general feature of bacteria induced by environmental stress.

5.2.2 Regulation of membrane fluidity in response to cold

The ability to cope with temperature downshift must be accompanied by a number of changes in response to physical and biochemical alterations, including solubility, membrane fluidity, protein conformation and stability, and changes in gene expression [145, 146, 256-259]. In this study, under the cold stress, the balance of the membrane fluidity was changed, the membrane related genes were detected and high protein abundance was found in the functional cluster of proteins regarding cell wall/membrane/envelope biogenesis. The differences in growth abilities of strains at low temperatures might be related to corresponding differences in enabling maintenance of cell fluidity.

5.2.2.1 Maintenance of membrane fluidity

As it was mentioned previously, the physical state of lipid bilayers is susceptible to the changes of temperature [260]. An abrupt temperature downshift leads to an increased proportion of unsaturated fatty-acid residues in microbial lipids, which results in a lowering of the melting point of the lipids and membrane fluidity [261, 262]. In our research, differences in membrane fluidity were shown in isolates with different growth abilities at low temperature. The membrane fluidity was maintained at the normal level in the isolates with higher growth abilities at low temperatures, but not in the isolate with lower abilities. Similar roles of membrane fluidity have been demonstrated in cold and other stresses in many bacteria [263, 264]. However, the fluidities are regulated by various mechanisms in different bacteria; e.g., *E. coli* [159], *B. subtilis* [265] and *Salmonella* [266, 267]

5.2.2.2 Regulation of lipid biosynthesis

As it was mentioned previously, in order to maintain membrane fluidity and functionality at low temperatures, bacteria modify their membrane physical properties by changing lipid composition [77]. The simplest way is to regulate the proportion of unsaturated fatty acids (UFAs) and saturated fatty acids (SFAs) in phospholipids [260, 268]. In many bacteria, temperature signals are processed to adjust enzyme activities or to activate genes to adapt the membranes to the new temperature. Among these, Des and LpxP are two fluidity-related enzymes mostly reported in response to cold. In *B. subtilis*, upon a drop in temperature, the Des protein is synthesized and desaturates the acyl chains of membrane phospholipids to increase the membrane fluidity [259, 265]. Furthermore, in *E. coli*, cold-induced acyltransferase LpxP helps to attach more unsaturated fatty acids (palmitoleate instead of laurate attached at normal temperature by LpxL) to lipid A, thus increasing membrane fluidity and lowering its phase transition temperature [258]. In our study, although slightly higher expression of *des* was found, induction of Des protein was not observed, which implies that the regulation function of Des in *Y. enterocolitica* might not be as important as in *B. subtilis.* Meanwhile, in our study, the expression of gene *lpxP* was significantly different in three isolates and the related proteins were induced in proteomic analysis. Therefore, membrane fluidity related to growth abilities in *Y. enterocolitica* was regulated by LpxM and LpxP in cold adaptation, which is similar to *E. coli*.

Our study implied the essential link between cold adaptation and membrane fluidity. The different regulation mechanism of fluidity have been investigated in other bacteria. In *L. monocytogenes*, the adaptation strategy relies on an increased amount of anteiso-form fatty acids and a reduction of the corresponding isoforms [269]. Accordingly, cold adaptation induced an up-regulation of the key enzyme in the biosynthesis of precursors of branched-chain fatty acids, and subsequent elongation steps of the fatty acid chain. These data clearly indicate an activation of lipid biosynthetic pathways [77]. The effect of low temperature on membrane lipid saturation has also been investigated in the cyanobacterium *Streptomyces platensis*. Total cellular lipids were analyzed and the different levels of membrane fatty acid unsaturation at 18 °C were compared with those at 22 °C. This result suggested that this bacteria has capacity to regulate its saturated fatty acid content, which could be a major factor in adaptability to low temperatures [270]. Therefore, membrane remodeling as one of the most common adaptations observed under cold stress has been seen in numerous psychrotrophs [271-274]. However, the modifications of fatty acid and membrane biosynthesis are various in different organisms to overcome decreased membrane fluidity at lower temperatures even in the bacteria in

very close species. For example, the differences were detected in low-temperature adaptation between *B. pertussis* and *B. bronchiseptica* by controlling plasticity of the membranes [275]. Alterations in membrane fatty acid composition in response to cold have also been documented in *Y. enterocolitica* and *Y. pseudotuberculosis* as well [276]. In *Y. pseudotuberculosis*, more total lipids and phospholipids are present in cells grown at 8°C than in cells grown at 37°C [277]. Saturated and cyclopropane fatty acids substantially increased and the unsaturated ones decreased when cultivation temperature was lowered in response to lower temperature in *Y. enterocolitica* [278]. However, the mechanism of cell membrane fluidity regulation and the relationship between cell membrane fluidity and growth ability at low temperatures remains unclear.

5.2.2.3 Regulation of outer membrane proteins

While exposed to the cold temperature, the membrane (as the selective barrier between living cells and their environment) plays a key role in cell viability. Based on our result, many membrane related genes were induced and expressed differently in response to cold. Firstly, four outer membrane protein assembly factors (encoded by yaeT, yfgL, dapX and yfiO) were detected. Significantly higher expression of *yaeT*, *yfgL* and *dapX*, and lower expression of *yfiO* were observed in isolates with low growth ability under cold stress. The related proteins were also found in proteomic analysis. The differential expression of these genes and proteins might be involved in fluidity regulation in response to cold. Similar functions of the outer membrane protein YaeT, DapX and YfgL were shown in *E. coli* and other bacteria according to previous researches in response to cold [279-284]. Moreover, the outer membrane phospholipase A (encoded by pldA), which is activated under various stress conditions and maintains the cell envelope integrity and permeabilization in response to temperature [285, 286]. The differential expressions of *pldA* gene was also detected in our research among isolates. This result suggested the possible involvement of outer membrane phospholipase A in fluidity maintenance under cold stress. Many other studies have shown the relationship between cold response and outer membrane regulation. For example, changes in these major outer membrane protein OmpA has been reported being affected by *hha* gene, which acts as a temperature-dependent modulator in *E. coli* [287]. In *Yersinia*, *ymoA* which is homologous to *hha* gene, was reported to serve the same function [288]. The impact of OmpR, OmpX, OmpF and OmpA of *Y. enterocolitica* associated with temperature changes has been demonstrated [137] [289].

In addition, according to the proteomic results, high protein abundance was detected in the functional cluster cell wall/membrane/envelope biogenesis in COG analysis. Therefore, it proved the function of fluidity in cold response and the mechanism involved in lipid biosynthesis and outer membrane regulation. However, their functions in cold response still remain to be elucidated in *Y. enterocolitica*.

5.2.2.4 Regulation of amino acid biosynthesis

According to previous study, activation of the amino acid biosynthetic pathways (i.e. histidine, valine, isoleucine, and tryptophan) was reported during growth under cold stress, which proved that the intracellular content of amino acids were part of the adaptive response enabling survival in bacteria under cold conditions [290-292]. In our research, the induced proteins participated in amino acid biosynthesis contribute to the regulation of membrane fluidity. Valine, leucine and isoleucine, as the branched-chain amino acids and the precursors for biosynthesis of iso- and anteiso-branched-chain fatty acids, were utilized to regulate the membrane fluidity in response to cold in certain

bacteria [293-295]. According to the proteomic results in this study, the induction of L*euA/B/C/D* proteins was only detected in isolate with low growth ability under cold stress, which implied the indispensable regulation of these branched-chain amino acids. Similarly, the association between isoleucine and leucine biosynthesis and cold response was also found in *E. coli* [296] and *Thermoanaerobacter tengcongensis* [297].

5.2.3 Regulation of cell motility in response to cold

Bacteria employs different strategies and mechanisms to withstand adverse environmental conditions. One of the most effective ways is to adjust its own movement. The diversity of motility mechanisms in response to stimuli allows them to migrate to optimal environments, incl. favorable temperatures [298].

5.2.3.1 Relationship between motility and cold response

Y. enterocolitica shows the highest motility at 25 °C and becomes non-motile at 37 °C due to the transcription prohibition of the main regulator of the filament assembly [299, 300]. In our study the motility of different isolates differed. All three tested strains showed motility at 28°C; however, at the temperature of 4°C, only the isolate with high growth ability at low temperatures was motile. This showed the effect of different temperature on motility. The influence of cold stress on motility was also reported in other bacteria, such as *B. subtilis* [301] and *V. cholerae* [302]. The effect of temperature on cell of motility is reported in relation with the transition between growth outside and inside the host in various pathogenic bacteria, such as *L. monocytogenes* [303], *B. bronchiseptica* [304], *Legionella pneumophila* [305] and *Actinobacillus pleuropneumoniae* [306]. Furthermore, the thermo-regulation of swarming motility has been reported in several bacteria including deep-sea bacterium *Shewanella piezotolerans* [307], plant pathogen *Pseudomonas syringae* and *Proteus* [308]. Moreover, the most common and best studied of all prokaryotic motility structures is the bacterial flagellum [309, 310]. The regulation of motility in some flagellated bacteria has been investigated and the related proteins have been reported sensing environment [311]. Furthermore, the physiological changes of flagella can be affected by the temperature. In *E. coli*, the flagella proteins are transiently induced following heat shock, salt, and acid stress or limitation of glucose [312] and the flagella biosynthesis and motility system were highly induced after a temperature downshift [313]. In *Campylobacter*, the expression of flagella genes is also known to be modulated by temperature [314]. Therefore, temperature is essential for cell motility.

5.2.3.2 Regulation of motility related proteins

According to the transcriptional analysis, the correlation between motility and growth ability was demonstrated in three isolates due to the different transcriptional changes of motility-related genes *fleC* (homologous to *fliC* and encoding Flagellin), *fliS* (putative cytoplasmic chaperone) and YE2848 (putative chemotaxis methyl-accepting transducer). The expression of motility-related genes (*fliS* and *fleC*) increased under cold stress at 4°C and the abundance of proteins was present differently in the “cell motility” group in isolates, which was consistent with the lower growth ability. Moreover, the proteins associated with motility and bacterial chemotaxis were identified and some cold-response proteins were only detected in isolates with high growth ability. For example, the Flg family, used for flagellar assembly and motility, are temperature- dependent in *E. coli* and other bacteria [315, 316]. The chemotaxis protein, Che family is essential for motility and cold response [297, 317]. Meanwhile, the Flagellin was detectable only in II7D but not in 44B in proteomic analysis (other related genes were not found). Since it is

also critical in motility and cold response in *Salmonella enterica* [318, 319], the involvement of motility in cold response might be demonstrated. Based on the results from transcriptional and proteomic analysis, the different induction of the motility-related genes and proteins among isolates with different growth behaviors indicated the close link between cell motility and growth ability in *Y. enterocolitica* [299]. However, a wide range of factors with complex mechanisms have been reported in regulating motility [19, 320, 321]. For example, the physiology of motility was reported to be changed via the chemotaxis system and the function of flagella in *E. coli* and *S.* Typhimurium in response to environmental signals [19, 322]. Based on comparative analysis of flagella regulatory cascades in *Enterobacteriaceae* family in terms of motility gene regulation by environmental factors are composed. As the members of *Enterobacteriaceae* family, *Y. enterocolitica* and *E. coli* meet the similarities of the flagella hierarchy including the identification of FlhD/FlhC as the master regulator [323], FliA as the sigma factor [324] and the genes within each operon [325]. However, there are also many specific mechanisms of flagella in *Y. enterocolitica*. For example, the arrangement of the operons along the chromosome and the environmental control of flagella regulon differs in *Y. enterocolitica* [326] and not all *Y. enterocolitica* flagella genes respond to the same environmental stimuli [326, 327]. Due to the complex regulation system of flagella, it is difficult to point out one single factor that generate cell motility. Therefore, how cell motility was affected by or contributed to the growth ability after cold response remains unclear.

5.2.4 Predicted cold-response regulation of *Y. enterocolitica*

According to the proteomic analysis in this study, a series of complex responses and processes changed rather than a simple increase or decrease happened at protein levels. Under cold stress, large changes were observed in protein synthesis, energy production and convention and carbohydrate transport and metabolism and also cell membrane parts and cell motility. In addition, proteins belonging to two-component system and ABC transporters were detected in cold response. The role of them in stress response has been reported in many bacteria [328, 329], they can be studied in detail to understand *Y. enterocolitica* response to low temperatures.

5.2.4.1 Two-component system

Two-component regulatory signaling system (TCS) is one of the major systems for bacteria in stress response. A TCS is constituted of a transmembrane sensor histidine kinase (HK) and a cytoplasmic response regulator (RR). The sensor domain of the HK accepts the signal of the specific stimulus or stress factor in the environment and the cognate RRs then catalyze the transfer of the phosphoryl group in response to the environmental stimulus [330-332]. In addition, TCSs have been reported to be involved in cold response of many bacteria. In this study, Che family was induced in response to cold and CheA/CheY working as the TCS was also found to be induced at low temperature in *Y. pseudotuberculosis* [333]. Moreover, the membrane phospholipid desaturase gene *des* was mentioned to be associated with membrane fluidity in this study. In *B. subtilis*, following cold shock, the transcription of *des* gene is induced by the TCS, DesK/DesR [334, 335]. However, to our knowledge, no DesK/DesR or casK/R homologue have been found in *Y. enterocolitica* [336-338]. Moreover, other TCSs has been found in cold response of many other foodborne pathogenic bacteria in *L. monocytogenes* (yycG/lisK) [336], *B. cereus* (CasK/R) [339], *L. monocytogenes* (LisK/LisR) [340, 341] and *Stenotrophomonas maltophilia* (LotS/LotR) [342].

According to the proteomic analysis, several predicted TCS related proteins were identified (listed in **Table 10**). However, the specific function of them has not been fully demonstrated. Thus, by concentrating the essential function of TCSs in stress experiments, relevant information may be unheeded in the further study.

Table 10: TCS related proteins identified in proteomic analysis

Protein Entry	Gene names	Protein names	Functional definition
A0A0E1NDU5	*arcA*	DNA-binding response regulator	Response regulators consisting of a CheY-like receiver domain and a winged-helix DNA-binding domain
A0A0U1HEG5	*arnB*	UDP-4-amino-4-deoxy-L-arabinose-oxoglutarate aminotransferase	Predicted pyridoxal phosphate-dependent enzyme apparently involved in regulation of cell wall biogenesis
A0A0E1NCT9	b4153	Succinate dehydrogenase iron-sulfur subunit	Succinate dehydrogenase/fumarate reductase, Fe-S protein subunit
A0A0E8MQY1	*bvgA*	Putative two-component response regulator	Response regulator containing a CheY-like receiver domain and an HTH DNA-binding domain
A0A0E1NK07	*cheB*	Chemotaxis response regulator protein-glutamate methylesterase	Chemotaxis response regulator containing a CheY-like receiver domain and a methylesterase domain
A0A0E8LV00	*cheD*	Methyl-accepting chemotaxis protein	Methyl-accepting chemotaxis protein
A0A0E1NG03	*cheY*	Chemotaxis regulatory protein CheY	CheY-like receiver
A0A0U1HH06	*citE*	Putative citrate lyase beta chain	Citrate lyase beta subunit
Q79RU4	*crp*	Cyclic AMP receptor protein	cAMP-binding proteins - catabolite gene activator and regulatory subunit of cAMP-dependent protein kinases
A0A0E8LXM1	*cydA*	Cytochrome D ubiquinol oxidase subunit I	Cytochrome bd-type quinol oxidase, subunit 1
A0A0F6ZK31	*degP*	Periplasmic serine endoprotease DegP-like	Trypsin-like serine proteases, typically periplasmic, contain C-terminal PDZ domain
A0A0U1HK63	*dnaA*	Chromosomal replication initiator protein DnaA	ATPase involved in DNA replication initiation
A0A0F6ZK28	*flgM*	Anti-sigma-28 factor FlgM	Negative regulator of flagellin synthesis (anti-sigma28 factor)
A0A0U1HI17	*fliC1*	Flagellin	Flagellin and related hook-associated proteins
A0A0U1HM52	*fliC2*	Flagellin	Flagellin and related hook-associated proteins
A0A0U1HFJ9	*frdA*	Fumarate reductase flavoprotein subunit	Succinate dehydrogenase/fumarate reductase, flavoprotein subunit

A0A0E8M398	*glnA*	Glutamine synthetase	Glutamine synthetase
A0A0E1NFB9	*glnB*	Nitrogen regulatory protein P-II 1 (Transcriptional regulator)	Nitrogen regulatory protein PII
A0A0N9KNE5	*glnF*	RNA polymerase sigma-54 factor	DNA-directed RNA polymerase specialized sigma subunit, sigma 54 homolog
A0A0U1HGN2	*gltI*	Glutamate Aspartate periplasmic binding protein GltI	ABC-type amino acid transport/signal transduction systems, periplasmic component/domain
A0A0E8LZT5	*gltL*	Putative glutamate/aspartate ABC transporter ATP-binding protein	ABC-type polar amino acid transport system, ATPase component
A0A0F6WYJ2	*mdtE*	Multidrug resistance protein MdtA	Membrane-fusion protein
A0A0U1HJG4	*meoA2*	Outer membrane porin protein C	Outer membrane protein (porin)
A0A0U1HJT3	*meoA3*	Outer membrane porin protein C	Outer membrane protein (porin)
A0A0F6WYX5	*ompF*	Putative outer membrane porin F protein	Outer membrane protein (porin)
A0A0E1NA84	*ompR*	DNA-binding response regulator	Response regulators consisting of a CheY-like receiver domain and a winged-helix DNA-binding domain
A0A0E1NCS5	*phoP*	DNA-binding transcriptional regulator PhoP	Response regulators consisting of a CheY-like receiver domain and a winged-helix DNA-binding domain
A0A0T9S2Z7	*phoS*	Phosphate-binding protein PstS	ABC-type phosphate transport system, periplasmic component
A0A0U1HBU6	*qseF*	Two-component system response regulator	Response regulator containing CheY-like receiver, AAA-type ATPase, and DNA-binding domains
A0A0E8IYW6	*tolC*	Outer membrane channel protein	Outer membrane protein
A0A0U1H7P4	*torC*	Cytochrome c-type protein	Nitrate/TMAO reductases, membrane-bound tetraheme cytochrome c subunit
A0A0U1HGP8	*trg*	Putative methyl-accepting chemotaxis protein	Methyl-accepting chemotaxis protein
A0A0E8KNA6	*uvrY*	Response regulator	Response regulator containing a CheY-like receiver domain and an HTH DNA-binding domain

5.2.4.2 ABC transporter

Bacterial cells need a kind of membrane barrier when exposed to external environments keeping the level of certain ions, metabolic intermediates and macromolecules. Therefore, during environmental adaptation, efficient transport systems have been developed to allow essential ions and metabolites enter the cell and other compounds leave it. The ATP-binding cassette (ABC) transporters, which constitute ATP-powered transporters, have a large of members in all organism. For example, it has 79 ABC transporters in *Escherichia coli* and 29 in *Saccharomyces cerevisiae* [343, 344]. In our research, 32 ABC transporters were found based on the proteomic analysis under cold stress (listed in **Table 11**), which implied the essential role of ABC transporters in cold response. The ABC transporters have been investigated in temperature-dependent situation in many other bacteria. For example, TliDEF, from *P. fluorescens* SIK W1, mediates the secretion of its cognate lipase in a temperature-dependent secretion manner [345]. The transcriptome data in *V. parahaemolyticus* revealed that the expression of the ABC transporter genes were significantly altered when it was grown at 10 °C [346]. In *Y. pseudotuberculosis*, the strong connection between the genes involved in ABC transporters and the cold response was mentioned in *Y. pseudotuberculosis* in controlling its growth during cold storage in food [347]. Hence, ABC transporters have received considerable attention recently because they are associated with many important biological processes. In contrast to the situation in other organisms, studies of *Y. enterocolitica* ABC proteins are still at an early stage. Although the general organization of the ABC gene family of *Y. enterocolitica* and its association with cold response haven't been well proved, the large variety of multiple physiological roles makes ABC transporters at the center of interest for stress response. According to our proteomic analysis, the predicted ABC transporters were observed.

One of the most studied ABC exporters in bacteria is the lipid flippase MsbA [348, 349]. MsbA is located in the inner membrane of Gram-negative bacteria, where it transports lipid A from the inner to the outer leaflet [349, 350]. As an important ABC exporter in bacteria, it proved the potential function of ABC transporter in cold response. In *E. coli*, this function has been well demonstrated and the *msbA* gene is essential for bacterial viability at all temperatures. In addition, the *msbA* gene was identified together with *htrB* gene, which affected growth and viability at high temperature [351-353]. According to our observation, the lipid A biosynthesis is essential for the membrane regulation in cold response in *Y. enterocoiltica* and the acyltransferase belong to LpxL/LpxM/LpxP family were found.

Table 11: ABC- transporter related proteins identified in proteomic analysis

Protein Entry	Gene names	Protein names	Functional definition
A0A0U1HFQ6	*argT*	Amino acid-binding periplasmic protein	ABC-type amino acid transport/signal transduction systems, periplasmic component/domain
A0A0E8HU69	*artJ*	periplasmic arginine-binding protein	ABC-type amino acid transport/signal transduction systems, periplasmic component/domain
A0A0U1H7W1	*artP*	Arginine transporter ATP-binding subunit	ABC-type arginine transport system, ATPase component
A0A0F6ZGJ8	*cysP*	Thiosulfate transporter subunit	ABC-type sulfate transport system, periplasmic component
A0A0E1NH39	*dppA*	Dipeptide transport protein	ABC-type dipeptide transport system, periplasmic component
A0A0U1HB05	*fepB*	Iron-enterobactin transporter periplasmic binding protein	ABC-type Fe2+-enterobactin transport system, periplasmic component
A0A0U1HJR7	*fliY*	Cystine transporter subunit	ABC-type amino acid transport/signal transduction systems, periplasmic component/domain
A0A0E1NKJ4	*glnH*	Glutamine ABC transporter periplasmic protein	ABC-type amino acid transport/signal transduction systems, periplasmic component/domain
A0A0E8JUV2	*glnQ*	Glutamine ABC transporter ATP-binding protein	ABC-type polar amino acid transport system, ATPase component
A0A0U1HGN2	*gltI*	Glutamate Aspartate periplasmic binding protein	ABC-type amino acid transport/signal transduction systems, periplasmic component/domain
A0A0E8LZT5	*gltL*	Putative glutamate/aspartate ABC transporter ATP-binding protein	ABC-type polar amino acid transport system, ATPase component
A0A0E1NFM5	*hisJ*	Histidine-binding periplasmic protein	ABC-type amino acid transport/signal transduction systems, periplasmic component/domain
A0A0E8GLB0	*lptG*	Lipopolysaccharide ABC transporter permease	ABC-type permeases
A0A0U1H7V7	*macB*	Macrolide export ATP-binding/permease protein MacB	ABC-type antimicrobial peptide transport system, permease component
A0A0U1HK35	*malK*	Maltose/maltodextrin import ATP-binding protein MalK	ABC-type sugar transport systems, ATPase components
A0A0U1HA52	*metN*	Methionine import ATP-binding protein MetN	ABC-type metal ion transport system, ATPase component
A0A0E1NIJ4	*metQ*	Lipoprotein	ABC-type metal ion transport system, periplasmic component/surface antigen

A0A0U1HDP9	*mglB*	Galactose-binding protein	ABC-type sugar transport system, periplasmic component
A0A0U1HE62	*modA*	Molybdate transporter periplasmic protein	ABC-type molybdate transport system, periplasmic component
A0A0U1HFV5	*mppA*	Putative periplasmic murein peptide-binding protein	ABC-type oligopeptide transport system, periplasmic component
A0A0E8JAK7	*peb1A*	periplasmic solute-binding protein	ABC-type amino acid transport/signal transduction systems, periplasmic component/domain
A0A0T9S2Z7	*phoS*	Phosphate-binding protein PstS	ABC-type phosphate transport system, periplasmic component
A0A0F6SWD0	*potF*	Putrescine-binding periplasmic protein	ABC-type Spermidine/putrescine-binding periplasmic protein
A0A0E8MBN4	*proV2*	Glycine betaine/L-proline transport ATP-binding protein	ABC-type proline/glycine betaine transport system, ATPase component
A0A0T9S1I3	*proX*	Glycine betaine transporter periplasmic subunit	ABC-type proline/glycine betaine transport systems, periplasmic components
A0A0U1HMA3	*rbsB*	D-ribose transporter subunit RbsB	ABC-type sugar transport system, periplasmic component
A0A0U1H9P3	*thiB*	Thiamine transporter substrate binding subunit	ABC-type thiamine transport system, periplasmic component
Q692K7	*wzt*	Lipopolysaccharide transport system ATP-binding protein	ABC-type polysaccharide/polyol phosphate transport system, ATPase component
A0A0U1HB62	*yclQ*	Putative iron transport protein	ABC-type enterochelin transport system, periplasmic component
A0A0E8KGP9	*yesO*	Putative sugar-binding protein	ABC-type sugar transport system, periplasmic component
A0A0E1NC09	*yhbG*	Putative ABC transporter ATP-binding protein YhbG	ABC-type (unclassified) transport system, ATPase component
A0A0F6ZJ75	*znuA*	High-affinity zinc transporter periplasmic protein	ABC-type Zn2+ transport system, periplasmic component/surface adhesion

CHAPTER 6 SUMMARY

The first part of the thesis determined the prevalence of *Y. enterocolitica* in seafood. The presumptive *Y. enterocolitica* isolates were analyzed by biotyping, serotyping and antimicrobial susceptibility testing. The total prevalence of *Y. enterocolitica* in seafood samples was 2.7% (6/220). All isolates belonged to biotype 1A and three isolates were identified as serotype O:8, one isolate as O:5,27, while two samples did not belong to the investigated serotypes. Most isolates contained the virulence-associated genes *ail*, *inv* and *ystB* and the isolates were resistant to cephalothin (83.3%), amoxicillin (83.3%) and ampicillin (50.0%). The results indicate that seafood might be a potential source of infection by *Y. enterocolitica*.

Since *Y. enterocolitica* (as psychrotrophic bacterium) is able to multiply at low temperatures, information about the underlying mechanisms is needed. However, information about *Y. enterocolitica* cold response is still scarce. In this study, strain specific growth profiles at 4°C were found and the cold response was investigated by combining phenotypic, gene expressional and proteomic approaches. The expressional changes of the cold-response genes showed that the ability to survive under cold stress depends on the transient induction and effective repression of cold-shock genes and the resumption of gene expression in other non-cold shock genes. Global proteomic analysis with label free quantification was firstly used in *Y. enterocolitica* cold response to indicate general cold response and resistance mechanism. Additionally, the isolate specific differences at transcriptional levels in motility- and fluidity- related genes contribute to our understanding of cold response regulated by motility and fluidity in *Y. enterocolitica*. By combining different approaches, cold response was described systematically, providing a better understanding of the physiological processes of *Y. enterocolitica* in response to cold stress.

CHAPTER 7 ZUSAMMENFASSUNG

Prävalenz von *Yersinia enterocolitica* in den Meeresfrüchten im Einzelhandel und die isolatspezifische Kältereaktion bei transkriptionellen, proteomischen und membranphysiologischen Veränderungen

Die Prävalenz von Y. enterocolitica in Meeresfrüchten ist in den ersten Teil der Arbeit bestimmt. Die mutmaßlichen Y. enterocolitica-Isolate wurden durch Biotypisierung, Serotypisierung und antimikrobielle Empfindlichkeitstests analysiert. Die Gesamtprävalenz von Y. enterocolitica in Meeresfrüchteproben betrug 2,7% (6/220). Alle Isolate gehörten zum Biotyp 1A und drei Isolate wurden als Serotyp O: 8 identifiziert, ein Isolat als O: 5,27 identifiziert, jedoch gehörten zwei Proben nicht zu den untersuchten Serotypen. Die meisten Isolate enthielten die Virulenz-assoziierten Gene ail, inv und ystB und die Isolate waren resistent gegen Cephalothin (83,3%), Amoxicillin (83,3%) und Ampicillin (50,0%). Die Ergebnisse zeigen, dass Meeresfrüchte eine potenzielle Infektionsquelle für Y. enterocolitica sein könnten.

Aus dem Grund, dass sich Y. enterocolitica (als psychotropes Bakterium) bei niedrigen Temperaturen vermehren kann, sind Informationen über die zugrunde liegenden Mechanismen erforderlich. Allerdings sind die Informationen über die Erkältungsreaktion von Y. enterocolitica noch nicht gut verstanden. In dieser Studie wurden stammspezifische Wachstumsprofile bei 4 ° C gefunden und die Kälteantwort durch die Kombinationen von phänotypischen, Genexpressions- und proteomischen Ansätzen untersucht. Die Expressionsänderungen der Kaltantwortgene zeigen, dass die Fähigkeit, unter Kältestress zu überleben, von der folgenden Faktoren abhängt, wie die vorübergehenden Induktion und wirksamen Unterdrückung der Kälteschockgenen und der Wiederaufnahme der Genexpression in anderen Nicht-Kälteschockgenen. Die globale Proteomanalyse mit markierungsfreier Quantifizierung wurde zuerst bei der Kälteantwort von Y. enterocolitica verwendet, um die allgemeine Kälteantwort und den Resistenzmechanismus anzuzeigen. Darüber hinaus tragen die isolatspezifischen Unterschiede auf Transkriptionsebene in Motilitäts- und Fluiditätsgenen zu unserem Verständnis über die Kälteantwort in Y. enterocolitica, die durch Motilität und Fluidität reguliert werden kann. Durch die Kombination verschiedener Ansätze wurde die Kälteantwort systematisch beschrieben, wodurch wir die physiologischen Prozesse von Y. enterocolitica als Reaktion auf Kältestress besser verstehen können.

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SELECTIVE PUBLICATIONS

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SELBSTÄNDIGKEITSERKLÄRUNG

Ich erkläre, dass ich die vorliegende Dissertation selbständig, ohne unzulässige fremde Hilfe und nur unter Verwendung der angegebenen Literatur und Hilfsmittel angefertigt habe.

Berlin, den 30.06.2020

Chenyang Li

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