

**Aus dem Institut für Lebensmittelhygiene
des Fachbereichs Veterinärmedizin
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Gene expression profiles of *Vibrio parahaemolyticus*

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Lu Meng
aus Xuzhou, China**

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Erster Gutachter: Univ.-Prof. Dr. Thomas Alter

Zweiter Gutachter: PD Dr. Burkhard Malorny

Dritter Gutachter: Univ.-Prof. Dr. Uwe Rösler

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天将降大任于是人也，
必先苦其心志，劳其筋骨，
饿其体肤，空乏其身，
行拂乱其所为，
所以动心忍性，曾益其所不能。

——孟子

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List of Abbreviations

<i>ahpC</i>	alkyl hydroperoxide reductase subunit C (gene)
AhpC	alkyl hydroperoxide reductase subunit C (protein)
approx.	approximately
<i>C.</i>	<i>Campylobacter</i>
CFU	colony forming unit
CTC	5-cyano-2,3-ditoly tetrazolium chloride
DAPI	4,6-diamino-2-phenyl indole
DFA-DVC	modified fluorescent antibody-direct viable count
DVC	direct count of viable bacterial cells
<i>E.</i>	<i>Escherichia</i>
ECF	extracytoplasmic function factors
EMA	ethidium monoazide
FISH	Fluorescence <i>in situ</i> hybridization
FITC	fluorescein isothiocyanate
GASP	growth advantage in stationary-phase
HeLa	Henrietta Lacks
IHF	integration host factor
INT	indophenyl-nitrophenyl-phenyltetrazolium chloride
KP+	Kanagawa phenomenon positive
KP-	Kanagawa phenomenon negative
LB	Luria-Bertani
log ₂ fc	log ₂ fold change
MALDI-ToF/MS	matrix-assisted laser desorption ionization time-of-flight mass spectrometry
MMR	methyl-directed mismatch repair
mRNA	messenger RNA
MSHA	mannose-sensitive haemagglutinin
OMPs	outer membrane proteins
<i>P.</i>	<i>Pseudomonas</i>
PCR	polymerase chain reaction
ppGpp	guanosine 3',5'-bis(diphosphate)
pppGpp	guanosine 3'-diphosphate, 5'-triphosphate
(p)ppGpp	together of ppGpp and pppGpp
PTS	phosphotransferase system
QS	quorum sensing
qPCR	Real-time quantitative polymerase chain reaction
RIN	RNA Integrity Number
ROS	reactive oxygen species
Rpfs	resuscitation-promoting factors
<i>rpoS</i>	RNA polymerase sigma factor S
RT-qPCR	reverse transcription quantitative polymerase chain reaction

List of Abbreviations

rRNA	ribosomal-RNA
<i>S.</i>	<i>Salmonella enterica</i> subsp. <i>enterica</i>
siRNA	short interfering RNA
t-BOOH	tert-butyl hydroperoxide
TCA	tricarboxylic acid
TCBS	thiosulfate citrate bile salts sucrose agar culture medium
<i>tdh</i>	thermostable direct hemolysin (gene)
TDH	thermostable direct hemolysin (protein)
<i>trh</i>	thermostable direct hemolysin-related hemolysin (gene)
TRH	thermostable direct hemolysin-related hemolysin (protein)
tRNA	transfer-RNA
T3SS	type three secretion system
T6SS	type six secretion systems
<i>V.</i>	<i>Vibrio</i>
VBNC	viable but non-culturable

Chapter 1 Introduction

Vibrio (*V.*) spp. are bacteria, which cause, or are associated with, human infections worldwide (FAO/WHO, 2011). Over 80 *Vibrio* spp. are described (ALTER, 2012; OLIVER *et al.*, 2013), with *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* as the most important species to human health. Moreover, *V. parahaemolyticus* is a crucial reason of bacterial gastroenteritis caused by seafood consumption in Asian countries (JOSEPH *et al.*, 1982; SU and LIU, 2007; WONG *et al.*, 2000a). On account of *in vivo* conditions in foods, e.g. low nutrients and pH value, it can be hypothesized that the majority of *V. parahaemolyticus* cells are in the stationary phase.

Bacteria can enter the stationary phase when nutrients are insufficient to maintain a steady growth. Stationary phase is the common phase for bacteria survival in the environment because a low concentration of nutrients is usual in environments (KOLTER *et al.*, 1993; NAVARRO LLORENS *et al.*, 2010). In the stationary phase, cells are metabolically active and undergo cell division without further increase in the number of cells (KOLTER *et al.*, 1993). *In vivo*, *V. parahaemolyticus* cells are also forced in the viable but non-culturable (VBNC) state for survival. The VBNC state is a special state which bacterial cells are alive but have lost the capability of growing in suitable media (OLIVER, 2000b). The VBNC state is referred to as a dormant state (COLWELL, 2000) enabling survival of especially non-sporulating bacteria in unfavorable or even hostile environments. Until now at least 85 bacteria species have been found to exist in the VBNC state (LI *et al.*, 2014). Several alterations occur during the shift of bacterial cells into the VBNC state, such as cell shape changes from rod to coccoid but maintains apparent cell integrity, cross-linking in cell wall increases as well as metabolic activity reduces (FAKRUDDIN *et al.*, 2013). In this study the alteration of gene expression in *V. parahaemolyticus* in the early stationary phase and the VBNC state was investigated. These data could help to investigate new detection methods to prevent the outbreak of gastroenteritis caused by *V. parahaemolyticus*.

1.1 Taxonomy of *Vibrio*

Vibrio belong to the Phylum of *Proteobacteria*, the Class of *Gammaproteobacteria*, the order of the *Vibrionales* and the family *Vibrionaceae* (THOMPSON *et al.*, 2004; THOMPSON and SWINGS, 2006). Up to now over 80 *Vibrio* species have been reported (ALTER, 2012; OLIVER *et al.*, 2013). At least 12 species infect humans as pathogenic agents (Table 1-1). Among them, eight species belonging to *Vibrio* Genus (*V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *V. mimicus*, *V. metschnikovii*, *V. hollisae*, *V. fluvialis*, and *V. furnissii*) are occasionally or frequently isolated from gastroenteritis cases and associated with food, while 6 species (*V. vulnificus*, *V. harveyi*, *V. alginolyticus*, *V. damsela*, *V. metschnikovii*, and *V. cincinnatiensis*) are mainly isolated from extra-intestinal infections (DEPAOLA and NISHIBUCHI, 2005; FDA, 2005; SCVPH, 2001). Of these 12 *Vibrio* species, the most important human pathogens are *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*.

Table 1-1: Pathogenic *Vibrio* spp. (modified from ALTER, 2012)

Family	Genus	Species
Vibrionaceae	<i>Vibrio</i>	<i>V. alginolyticus</i>
		<i>V. cholerae</i>
		<i>V. cincinnatiensis</i>
		<i>V. damsela</i> (<i>Photobacterium damsela</i>)
		<i>V. furnissii</i>
		<i>V. fluvialis</i>
		<i>V. harveyi</i> (<i>carchariae</i>)
		<i>V. hollisae</i> (<i>Grimontia hollisae</i>)
		<i>V. metschnikovii</i>
		<i>V. mimicus</i>
		<i>V. parahaemolyticus</i>
		<i>V. vulnificus</i>

1.2 History of *Vibrio*

Vibrio was first described in 1854 by an Italian physician (PACINI, 1854) and then was acknowledged with the name “*Vibrio cholerae* Pacini 1854” in 1965 (FRERICHS, 2001). *V. parahaemolyticus* was first reported in a major gastroenteritis outbreak in Japan in 1950 (FUJINO *et al.*, 1953). Since then, it is found to be a crucial agent of causing human diarrhea, especially in Asian countries (JOSEPH *et al.*, 1982; SU and LIU, 2007; WONG *et al.*, 2000a).

In early 1996, there was a rapid increase in the incidence rate of *V. parahaemolyticus* in India associated with the O3:K6 serotype (OKUDA *et al.*, 1997). From then on, strains belonging to the O3:K6 serotype have been found causing several outbreaks throughout the world (SERICHANTALERGS *et al.*, 2007; SUZUKI *et al.*, 1994; VELAZQUEZ-ROMAN *et al.*, 2014). An analysis of 178 USA isolates from several seafood sources (e.g. oysters) has shown 27 different serological types, and most clinical isolates from the Pacific Coast are also serotype O3:K6 (DEPAOLA *et al.*, 2003a; DEPAOLA *et al.*, 2003b). Moreover, *V. parahaemolyticus* serotype O3:K6 was detected and isolated from clinical patients in France, Spain and Italy in 2005 (MARTINEZ-URTAZA *et al.*, 2005; OTTAVIANI *et al.*, 2010). Serotypes O4:K68, O1:K25, O1 KUT (untypeable), O1:K41, O1:K42 and O6:K18 have been also attracting more attention because of the pandemic potential (CHOWDHURY *et al.*, 2000; HARA-KUDO *et al.*, 2003; MATSUMOTO *et al.*, 2000; YEUNG *et al.*, 2002). All cases were determined to relate with contaminated seafood.

1.3 *Vibrio parahaemolyticus*

1.3.1 Morphology

Vibrio species are gram-negative, aerobic or facultative anaerobic, curved rod-shaped and small (0.5-0.8 µm in width x 1.4-2.6 µm in length) bacteria. They own one or more polar flagella which facilitate their high motility in liquid media (ALTER, 2012; DRAKE *et al.*, 2007; McLAUGHLIN, 1995). *Vibrio* spp. mainly exist in seawater and seafood; however, the presence and distribution depend on a series of environmental factors (e.g. temperature, pH, salinity, pressure and nutrients). For example, *Vibrio* spp. are apt to grow in warm waters (around 20°C) (THOMPSON *et al.*, 2009), and tolerate a wide range of salinities (ALTER, 2012).

V. parahaemolyticus grows at concentrations of NaCl between 1% and 8%, with best growth at 2%-4% (SAKAZAKI *et al.*, 2006). Unlike *V. cholerae* and *V. mimicus*, the growth of *V. parahaemolyticus* is inhibited when the NaCl concentration falls below 0.5%, with no growth in distilled water or over 10.5% NaCl (LEE, 1972; NAUGHTON *et al.*, 2009; ONGAGNA-YHOMBI and BOYD, 2013). The optimal growth temperature of *V. parahaemolyticus* is between 5-43 °C with an optimal range of 30-35 °C. Temperatures below 5 °C significantly decrease the amount of culturable cells (FERNANDEZ-PIQUER *et al.*, 2011; ICMSF, 1996). In

addition, the pH for *V. parahaemolyticus* growth ranges from 4.8 to 11 with an optimal range of 7.8-8.6 (ICMSF, 1996), and pH lower than 4 is lethal to *V. parahaemolyticus* (WONG et al., 2000b).

Similar to other *Vibrio* pathogenic species, two chromosomes are present in *V. parahaemolyticus*. The sizes of them are 3.2Mb (chromosome 1) and 1.9Mb (chromosome 2) (YAMAICHI et al., 1999). Moreover, both chromosomes are replicated once per cell cycle. Chromosome 1 contains most of the fundamental genes responding to growth and viability. More genes required for transcriptional regulation and transportation are encoded on chromosome 2 as well as more species-specific genes. Studies have suggested that chromosome 2 might play a crucial role in adaptation to environmental change (BOYD et al., 2008; MAKINO et al., 2003; REEN et al., 2006).

One of the characteristics of *V. parahaemolyticus* is that its growth is very rapid. It only took 9 minutes in culture medium and 12 minutes in a host for one generation under suitable conditions (AIYAR et al., 2002; OLIVER et al., 2013). This fast growth could be resulting from a higher number of ribosomal-RNA (rRNA) and transfer-RNA (tRNA) genes in aquatic bacteria. In *V. parahaemolyticus* RIMD 2210633, for example, 126 genes encode tRNAs and eleven different 16S rRNA genes (REEN et al., 2006). Because of the high growth rate, this species can be deemed to an important cause of gastroenteritis associated with consumption of raw or undercooked seafood.

1.3.2 Classification

V. parahaemolyticus strains can be serotyped according to somatic O-antigens, as well as the capsular polysaccharide K-antigens (TWEDT et al., 1972) presence. There are 13 O (LPS) serogroups and more than 71 K (acidic polysaccharide) antigens presented (ALAM et al., 2009; KAYSNER and DEPAOLA, 2004), which results to a total of 75 different serovars that have been reported already. The first description of serotype O3:K6 uncovered the correlation between serotype and virulence. *V. parahaemolyticus* serotype O3:K6 is distributed worldwide and causes enterogastritis, while other serotypes, such as O4:K68, O1:K25, O1 KUT (untypeable), are reported to have molecular characteristics identical to that of the serotype O3:K6. These suggested that other serotypes could evolve from the original O3:K6 and can be defined as “serovariants” of the serotype O3:K6 isolate (CHOWDHURY et al., 2000; GONZALEZ-ESCALONA et al., 2008; NAIR et al., 2007). Up to now, at least 12 serotypes including serotype O3:K6 have been identified as pathogenic *V. parahaemolyticus* strains. Moreover, the serotype O3:K6 and all the serovariants are referred to as strains of the pandemic clone (CHEN et al., 2011; GONZALEZ-ESCALONA et al., 2008). The pandemic clone of *V. parahaemolyticus* has currently been identified in areas of Asia, Europe, Africa, and North and South America.

Unlike other *Vibrio* species, *V. parahaemolyticus* is able to produce a hemolysin. Many *V. parahaemolyticus* strains produce the thermostable direct hemolysin (TDH), which is initiating the infection in humans. These “Kanagawa phenomenon positive” (KP+, *tdh*+) *V. parahaemolyticus* strains cause hemolysis on blood agars but only 1%-2% of environmental strains are reported to encode *tdh* (COOK et al., 2002; DEPAOLA et al., 2000; MIYAMOTO et al., 1969). In contrast, some *V. parahaemolyticus* strains that lead to diarrhea are “Kanagawa phenomenon negative” (KP-, *tdh*-) strains (HONDA et al., 1988), and these strains produce a TDH-related hemolysin (TRH) instead of TDH. Besides its role as primary virulence factors, the TDH and TRH encoded by *tdh* and *trh* genes are also considered in classification.

1.3.3 Virulence mechanisms

The main virulence factors of *V. parahaemolyticus* are TDH and TRH. The virulence results from TDH produced by *V. parahaemolyticus* isolates (SHIRAI et al., 1990). TDH is composed

of two immunologically identical subunits, which are encoded via two non-identical *tdh* genes on chromosome 2, VPA1314 (*tdh2*) and VPA1378 (*tdh1*), sharing 97% identity (MAKINO *et al.*, 2003; TAKEDA *et al.*, 1978). However, there is a difference of seven amino acid residues in the protein (OLIVER *et al.*, 2013). Moreover, *tdh2* has a stronger promoter than *tdh1*, which leads to a higher expression. The expression of *tdh1* in *tdh+* strains is lower than 5% of the *tdh2* (NISHIBUCHI, 2003). Therefore, *tdh2* has a stronger effect on TDH protein than *tdh1* (MAKINO *et al.*, 2003; NISHIBUCHI and KAPER, 1995; OKUDA and NISHIBUCHI, 1998). TDH is named after its heat stability. Heating at 100 °C for 30 min only partly inactivates the hemolytic activity of TDH. TDH causes cytotoxic effects, such as lysing erythrocytes of various animals (LYNCH *et al.*, 2005). TDH produces pores in erythrocytes and intestinal cells, which increases vascular permeability in rabbit skin and stimulate fluid accumulation in the rabbit ileal loop (HARDY *et al.*, 2004; HONDA *et al.*, 1992; HUNTLEY *et al.*, 1993; NISHIBUCHI *et al.*, 1989; TAKEDA, 1982). Ion flux in myocardial tissue intestinal tract, rat erythrocytes, and human erythrocytes is altered (HUNTLEY *et al.*, 1993; SEYAMA *et al.*, 1977; TAKASHI *et al.*, 1982). TDH is also shown to induce chloride ion secretion in a rabbit model and further more using Ca^{2+} as an intracellular second messenger (RAIMONDI *et al.*, 1995; TAKAHASHI *et al.*, 2000). TDH is the first bacterial enterotoxin which has been confirmed to play an important role in the changes of intracellular calcium concentration and secretory activity.

V. parahaemolyticus tdh- strains rarely cause enterogastric outbreaks. Another hemolysin, TRH, a TDH-related hemolysin, is produced rather than TDH. TRH is encoded by *trh*, which can be classified into two subtypes, *trh1* and *trh2*. Both genes *trh1* and *tdh2* share 69% homology (KISHISHITA *et al.*, 1992). Moreover, TRH shares similar biological, structural and immunological characteristics with TDH (HONDA *et al.*, 1988; NISHIBUCHI *et al.*, 1989). However, TRH is thermolabile, and is inactivated at 60 °C for 10 min (HONDA *et al.*, 1988). Most clinical isolates encode either *tdh* or *trh*; both genes are encoded only in a small percentage (NISHIBUCHI and KAPER, 1995; SHIRAI *et al.*, 1990). In intestinal epithelial cells chloride ions (Cl⁻) secretion is increased by TRH, followed by the raise of intracellular calcium (TAKAHASHI *et al.*, 2000). Thus, TRH is another important virulence factor and may be the cause of diarrhea resulting from *tdh-* *V. parahaemolyticus* infections. Although 96.5% strains isolated from human patients are *tdh+* (SAKAZAKI *et al.*, 1968), only a small proportion of the total *V. parahaemolyticus* population in the environment are *tdh+* strains (JOSEPH *et al.*, 1982). In general, *tdh+* isolates are found to better adapt to the enteric canal, while *tdh-* strains own greater survival in natural environment (JOSEPH *et al.*, 1982).

Other factors in the genome sequence may play supplemental roles in pathogenic *V. parahaemolyticus*, although they are not directly related with clinical strains (Table 1-2). Many factors (e.g. chemotaxis, flagella, and iron acquisition systems) have been studied at the genomic level. Additionally, the type three secretion system (T3SS) of *V. parahaemolyticus* O3:K6 is of importance, which has two gene clusters (MAKINO *et al.*, 2003). T3SS1 system is encoded on chromosome 1, which participates in cytotoxicity in Henrietta Lacks (HeLa) cells (ONO *et al.*, 2006; PARK *et al.*, 2004). T3SS1 is encoded in all *V. parahaemolyticus* strains, whereas T3SS2 is present only in *tdh+* strains (MAKINO *et al.*, 2003). T3SS2 is encoded on chromosome 2, which shares the same pathogenicity island as *tdh*. The deletion of the T3SS2-related genes can lead to enterotoxigenicity lessening in rabbit ileal loops (PARK *et al.*, 2004). Moreover, type six secretion systems (T6SS) is complex and widespread gram-negative protein secretion systems (SILVERMAN *et al.*, 2012). At least 25% of all sequenced gram-negative bacteria encode T6SS (JANI and COTTER, 2010). T6SS is not only involved in pathogenicity and bacteria-host interactions, but also in symbiosis, interbacterial interactions and antipathogenesis (JANI and COTTER, 2010; RECORDS, 2011). As T3SS, T6SS gene clusters have been identified on both chromosomes in *V. parahaemolyticus* (IZUTSU *et al.*, 2008;

MAKINO *et al.*, 2003). T6SS1 is encoded on chromosome 1 and found mainly in clinical isolates, while T6SS2 is encoded on chromosome 2 and found in all *V. parahaemolyticus* strains that have been investigated so far (BOYD *et al.*, 2008; YU *et al.*, 2012). T6SS1 shows highest expression under warm marine-like conditions (in high salt media at 30 °C) and is repressed by quorum sensing (QS) master regulator OpaR, whereas T6SS2 is strong induced under low salt conditions and is positively regulated by OpaR (GODE-POTRATZ and MCCARTER, 2011; MA *et al.*, 2012). Both *V. parahaemolyticus* T6SSs contribute to adhesion to cultured cell monolayers (YU *et al.*, 2012). However, both of the T6SSs are not active at 37 °C, indicating that T6SSs maybe not directly relate with virulence but favor the survival of the pathogens via interbacterial competition in the marine environment (SALOMON *et al.*, 2013).

Table 1-2: Virulence factors and regulators of *V. parahaemolyticus* (modified from DEPAOLA and NISHIBUCHI, 2005)

	Virulence factor or regulator	Reference
Major virulence factors	Thermostable direct hemolysin (<i>tdh</i>)	NISHIBUCHI <i>et al.</i> (1992)
	TDH-related hemolysin (<i>trh</i>)	XU <i>et al.</i> (1994)
Other virulence factors	Type three secretion system (T3SS)	MAKINO <i>et al.</i> (2003)
	Type six secretion system (T6SS)	Makino <i>et al.</i> (2003)
	Chemotaxis and motility	KIM and MCCARTER (2000); MCCARTER and WRIGHT (1993)
	Iron acquisition systems (<i>pvuA</i> , <i>psuA</i>)	FUNAHASHI <i>et al.</i> (2002)
	Pilus	NAKASONE and IWANAGA (1990)
	Cell-associated hemagglutinin	NAGAYAMA <i>et al.</i> (1995)
	Thermostable hemolysin	TANIGUCHI <i>et al.</i> (1990)
	Thermolabile hemolysin (<i>tlh</i>)	SHINODA <i>et al.</i> (1991); TANIGUCHI <i>et al.</i> (1986)
Virulence regulators	Metalloproteases (<i>prtVp</i> , <i>vppC</i>)	KIM <i>et al.</i> (2002); LEE <i>et al.</i> (1995)
	ToxR (<i>toxR</i>)	LIN <i>et al.</i> (1993)
	Regulator of iron acquisition system (<i>fur</i>)	YAMAMOTO <i>et al.</i> (1997)

1.3.4 *V. parahaemolyticus* in seafood

Since *V. parahaemolyticus* is a bacterium in estuarine and coastal waters, it is frequently isolated from various samples throughout the world, especially from seafood (e.g. fish, oysters, clams, mussels, crabs, shrimp and lobsters) during summer (FAO/WHO, 2011; HLADY, 1997; KAYSNER and DEPAOLA, 2004). The prevalence of *V. parahaemolyticus* in examined seafood samples is found to be as high as 96.5% (195/202) (THU TRA *et al.*, 2015). Consuming raw fish containing *V. parahaemolyticus* is reported to be the main cause of food poisoning in Asia, especially in Japan and Taiwan (OKABE, 1974; PAN *et al.*, 1996; PAN *et al.*, 1997). In contrast, the cases of diarrhea in the USA and Europe are caused by consumption of raw oysters and other bivalves (MARTINEZ-URTAZA *et al.*, 2005; NOLAN *et al.*, 1984; RIPPEY, 1994). Studies of *Vibrio* species prevalence in bivalve molluscs, crustaceans and seawater worldwide are listed in Table 1-3. A three-year study in the USA has shown that 46% of 716 seafood samples are positive for *V. parahaemolyticus* which were mainly oysters, clams and crabs (HACKNEY *et al.*, 1980).

Table 1-3: Prevalence of *V. parahaemolyticus* and *Vibrio* species in seafood (modified from URMERSBACH, 2014)

Sample type (Number)	Country	Year	<i>Vibrio</i> spp. positive proportion	<i>Vp</i> positive proportion	Reference
Bivalves (50)	Germany	2007-2008	92 %	20 %	JUDEK <i>et al.</i> (2008)
Bivalves (82)	Germany	2009-2010	50 %	15 %	RANDT <i>et al.</i> (2011)
Blue mussels (<i>Mytilus edulis</i>) (82)	Germany	2004-2005	74,4 %	39,5 %	LHAFI and KUHNE (2007)
Blue mussels (<i>Mytilus edulis</i>) (885)	Norway	2002-2004	n. t. ¹	10,3 %	BAUER <i>et al.</i> (2006)
Crustacean and shellfish (82)	Germany	2001	19,5 %	6,3 %	SIEFFERT and STOLLE (2002)
Fish and crayfish (710)	Germany	Unknown	17,7 %	29,4 %	LEHMACHER and HANSEN (2007)

Molluscan shellfish (173)	Japan	2001	n. t. ¹	95,8 %	HARA-KUDO <i>et al.</i> (2003)
Molluscan shellfish (350)	USA and Canada	1998-1999	n. t. ¹	72,9 %	COOK <i>et al.</i> (2002)
Mussels (<i>Mytilus Galloprovincialis</i>) (62)	Italy	1997-1998	48,4 %	3,3 %	RIPABELLI <i>et al.</i> (1999)
Mussels and seawater (726)	Italy	2 Years	46,9 %	10 %	CROCI <i>et al.</i> (2001)
Oysters (156)	USA	1999-2000	100 %	100 %	DEPAOLA <i>et al.</i> (2003a)
Prawn and shellfish (338)	Germany	2008-2009	12,1 %	82,9 %	MESSELHAUSSER <i>et al.</i> (2010)
Seafood and fish (123)	Senegal	2007-2009	n. t. ¹	30,1 %	COLY <i>et al.</i> (2013)
Shellfish (60)	Chile	2008-2009	n. t. ¹	88,1 %	GARCIA <i>et al.</i> (2009)
Shellfish (147)	Italy	2011-2012	n. t.	40,8 %	SUFFREDINI <i>et al.</i> (2014)
Shrimp, crab and cockle (120)	Egypt	2011	n. t. ¹	41,7 % ³	ABD-ELGHANY and SALLAM (2013)
Shrimp (170)	Sri Lanka	2010-2011	95,1 % ²	91,2 % ²	KORALAGE <i>et al.</i> (2012)
Shrimp in inland ponds (16)	Thailand	2007-2009	100 %	37,5 %	YANO <i>et al.</i> (2014)

n. t., not tested; *Vp.*, *V. parahaemolyticus*

¹ the samples were examined only for the presence of *V. parahaemolyticus*.

² based on the examined area.

³ results via biochemical detection was 33.3% of the samples

1.4 The bacterial life cycle

1.4.1 Phases of bacterial growth cycle

The stages of bacterial growth can be artificially divided into several phases: lag phase (phase 1), exponential phase (phase 2), stationary phase (phase 3), death phase (phase 4) and extended or long-term stationary phase (phase 5) (Fig. 1-1). Finkel (2006) described that the bacterial life cycle comprised of five stages using CFU mL⁻¹ measurement of *Escherichia (E.) coli* growing under optimal laboratory conditions (37 °C, rich medium with agitation). However, in the environment it is difficult to distinguish the timing of the five phases due to many conditions (e.g. the species and environmental specificities). Under laboratory conditions bacterial cells shift from exponential phase into the stationary phase as soon as medium nutrients are exhausted. Only few cell divisions occur in the stationary phase. Waste products as the results of bacterial metabolism are accumulated, leading the bacteria into death phase. The definition of death is the loss of viable counts with standard plating assays (FINKEL, 2006). Up to 99% of the *E. coli* population enters death phase after three days incubation in Luria-Bertani (LB) medium (FINKEL, 2006). Surviving bacteria can maintain viability for long periods because of the nutrients released by the dead cells. These bacteria survive many months even years without supplemental nutrients after entering the long-term stationary phase (FINKEL and KOLTER, 1999; FINKEL *et al.*, 2000). In order to resist starvation, Gram-positive bacteria can produce dormant spores, whereas Gram-negative bacteria form resistant cells without dormancy (NAVARRO LLORENS *et al.*, 2010).

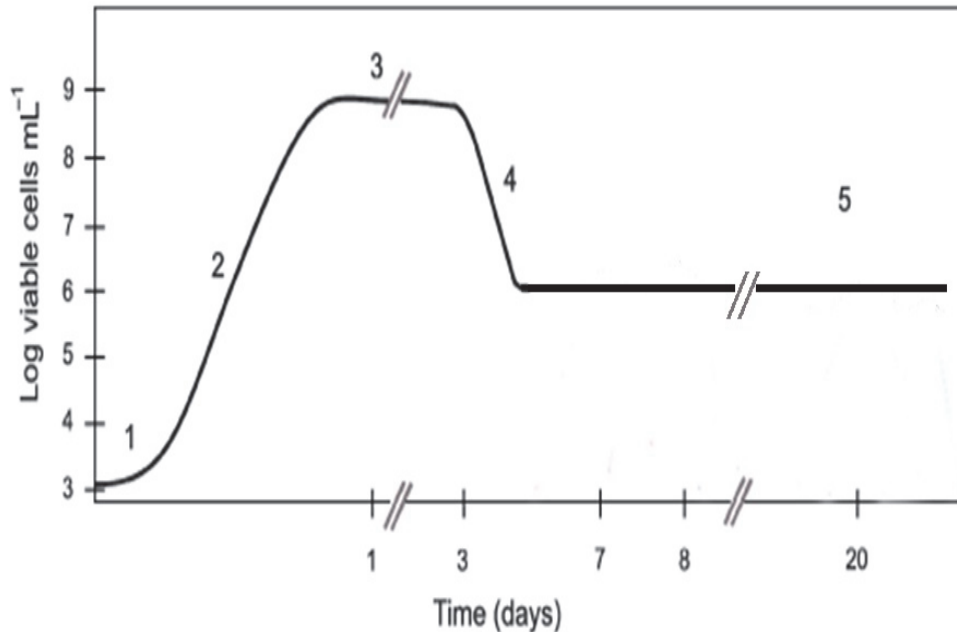


Fig. 1-1: The five phases of *E. coli* cells growth.

1, lag phase; 2, exponential phase; 3, stationary phase; 4, death phase; 5, extended or long-term stationary phase. (modified from FINKEL, 2006 and NAVARRO LLORENS *et al.*, 2010).

1.4.2 Exponential growth phase

The Lag phase is the first phase for bacterial cells to apperceive and adapt a new situation e.g. cells shifting into a new habitat. Once bacterial cells adapt the new habitat and start the cell division exponentially, they are in the exponential phase, also known as phase of logarithmic growth (log phase). The number of new bacteria appearing per unit time is proportional to the present population. The growth rate depends on bacterial species and the media used. During the exponential phase, many proteins involved in anabolic pathways showed twofold up-regulation (COHEN *et al.*, 2006). The majority of these induced proteins belong to functional categories of amino acid metabolism, (cellular) energy metabolism, protein biosynthesis, translation, as well as protein, lipid, and nucleotide biosynthesis. Some proteins involved in DNA replication and repair, as well as cells division are also up-regulated during the exponential phase. Glucokinase and phosphoglycerate mutase, enzymes crucial for energy generation, are induced more than twofold in the exponential phase. According to these up-regulated genes, the most significant reactions occurring in the exponential phase are metabolic pathways induced producing sufficient energy metabolites.

1.4.3 Stationary phase

Because oligotrophic environments are prevalent in the biosphere, bacteria are commonly growing in the condition of near-starvation (MORITA, 1997). Bacteria can enter the stationary phase when nutrients are decreasingly hampering the steady growth. Since low nutrients are common in environments, bacteria are forced to stay in the stationary phase. Thus, the stationary phase is the common phase for bacteria in the environment (KOLTER *et al.*, 1993; NAVARRO LLORENS *et al.*, 2010). In the stationary phase, cells are metabolically active and undergo cell division without further increase in the total number of cells (KOLTER *et al.*, 1993).

1.4.3.1 Characteristics of stationary phase

When bacterial cells enter the stationary phase a number of alterations happen, including e.g. morphology of cells, composition of cell membrane, metabolic processes, and genes expression (NAVARRO LLORENS *et al.*, 2010).

The dwarfing of bacterial cells is observed once they entered the stationary phase. Dwarfing occurs because of self-digestion, caused by starvation. It is referred to as a result of degradation of endogenous material, including cell envelope, especially the cytoplasmic membrane and cell wall (NAVARRO LLORENS *et al.*, 2010; NYSTROM, 2004). The reduction of cell division processes upon cells entering the stationary phase as well. The reduction of cell division increases the surface/volume ratio of cells, but it is not a starvation-induced activity (NYSTROM, 2004; NYSTROM and KJELLEBERG, 1989). The result of reduced cell division is the coccoid appearance of cells (NYSTROM, 2004). Both, the dwarfing and the reduced cell division induce bacterial morphological changes (NYSTROM, 2004). This coccoid shape is partly regulated by morphogene *bolA*, which is controlled by RpoS in all phases of bacterial growth (LANGE and HENGGE-ARONIS, 1991; SANTOS *et al.*, 2002).

The cell envelope protects bacterial cells from external damages. The changes of structures in the cell envelope were found, including composition of the peptidoglycan layer, lipopolysaccharides, the outer as well as the inner membranes (HUISMAN *et al.*, 1996). Although outer membrane protein synthesis is reduced (ALLEN and SCOTT, 1979), the cross-linking of outer membrane lipoproteins with the peptidoglycan layer is increased (HUISMAN *et al.*, 1996). Moreover, the thickness of peptidoglycan layer is increased as well (MENGIN-LECREULX and VAN HEIJENOORT, 1985). The concentration of lipopolysaccharides in outer membrane increases in the stationary phase (NAVARRO LLORENS *et al.*, 2010). A number of changes occur in the inner membrane as well. Unsaturated fatty acids are converted into cyclopropyl derivatives (CRONAN, 1968). Furthermore, a reduction of monounsaturated fatty acids occurs because polyunsaturated fatty acid concentrations increase (HUISMAN *et al.*, 1996). Bacterial cells shifting into the stationary phase, increase the relative concentrations of phosphatidylglycerol and phosphatidylethanolamine (CRONAN, 1968; HUISMAN *et al.*, 1996). All changes of the inner membrane result in a highly ordered structure and reduced fluidity (NYSTROM, 2004).

Cytoplasm alteration is accompanied with changes in the cell envelope. The nucleoid becomes condense in order to protect the DNA from the environmental stresses. A nonspecific DNA-binding protein, Dps, organizes the chromosome into a highly ordered, stable nucleoprotein complex within starvation. Dps is described in more than 130 bacterial species (NAIR and FINKEL, 2004). It is the most induced protein in *E. coli* during the induction of shifting into the stationary phase (ALMIRON *et al.*, 1992). Dps induction occurred not only by RpoS when cells transferred from exponential phase to the stationary phase but also by σ^{70} and OxyR exposed to oxidative stress in the exponential phase (ALMIRON *et al.*, 1992; ALTUVIA *et al.*, 1994). During the stationary phase *dps* induction results in protection from a large range of stressors, including oxidative stress, UV-light and gamma irradiation, iron and copper toxicity, thermal stress, and acid and base shock. The effect of Dps on DNA protection can be achieved through a combination of functions, such as direct Dps-DNA interactions to sequestering the chromosome into the highly stable biocrystal complex, reducing the production of oxidative radicals, and regulating the expression of genes that are required for stress responses of stationary phase cells (ILARI *et al.*, 2002; NAIR and FINKEL, 2004). The cell growth rate, protein synthesis, as well as rRNA and tRNA concentrations are reduced in stationary phase populations compared to cells in exponential phase (REEVE *et al.*, 1984). Moreover, the metabolism of carbohydrates, amino acids and phospholipids and transportation systems activity are also diminished (CLARK *et al.*, 2006). However, because of the synthesis of proteases and peptidases in the early stationary phase, the concentration of polypeptides was increased five times (GROAT *et al.*, 1986). A raised synthesis of glycolysis enzymes, pyruvate formate lyase, phosphotransacetylase and acetate kinase in catabolic activities during the stationary phase is important, which could be a defense metabolism to protect bacteria damage in starvation (NYSTROM, 2004; NYSTROM *et al.*, 1996). Tricarboxylic

acid (TCA) cycle enzymes are diminished in the stationary phase, indicating the repression of aerobic metabolism. The reduction of TCA cycle enzymes might block an uncontrolled utilization of endogenous reserves during autophagy, and moreover it might serve as a defense mechanism in starvation against the damage effects of reactive oxygen species which are produced by the respiratory chain (NYSTROM, 2004). Dimerization of the ribosomes, without a composition change, is induced because of the reduction of the rate of protein synthesis (YOSHIDA *et al.*, 2002). It is regulated by a ribosome modulation factor (WADA *et al.*, 2000). Dimerized ribosomes could be a way storing ribosomes during translational inactivity periods. Ribosome dimerization is shown to be reversed within 2 min with an addition of nutrients (YOSHIDA *et al.*, 2004).

In the stationary phase a wide production of secondary metabolites, antibiotics and toxins happens (NAVARRO LLORENS *et al.*, 2010). Microcins, are mainly produced during the stationary phase, are low-molecular-mass bacteriocins with a strong antibacterial activity against closely related bacteria and contribute to the regulation of microbial competitions within the intestinal microbiota (DUQUESNE *et al.*, 2007a; DUQUESNE *et al.*, 2007b). Microcins might play a key role in this phase, although the mechanism is still unclear. Moreover, the implications of some growth-dependent bacteriocins, which are highly induced during the stationary phase, need to be clarified.

To sum up, bacterial cells in the stationary phase show the following characteristics (NAVARRO LLORENS *et al.*, 2010):

- (i) Dwarfing cell size and changing cell shape to coccoid;
- (ii) Highly ordered cell envelope structure;
- (iii) Condensation of the nucleoid;
- (iv) Increased synthesis of catabolic enzymes;
- (v) Decreased protein synthesis while peptidases and/or proteases synthesis increases;
- (vi) Dimerization of ribosomes;
- (vii) Reduction of aerobic metabolism;
- (viii) Production of fermentative secondary metabolites.

1.4.3.2 Stationary phase regulators and effectors

The altered production of sigma factors enables a stationary bacterial population to become more adaptable and resistant to harmful environments. RpoS, a global stress response regulator, plays an important role in governing both entering the stationary phase and stress resistance (HENGGE-ARONIS, 2002). However, not only RpoS but also the whole sigma factor family plays a role in the stationary phase. Based on structural and functional criteria, sigma factors can be divided into the σ^{70} -family and the σ^{54} -family (PAGET and HELMANN, 2003). Since σ^{70} recognizes the promoters of most housekeeping genes in *E. coli* (GRUBER and GROSS, 2003), it plays a key role in bacteria. The σ^{70} -family can be further classified into four groups. Group 1 sigma factors, including σ^{70} , are essential for cell growth, while group 2 sigma factors are similar to σ^{70} but not fundamental for bacterial growth. Group 3 includes sigma factors that control heat shock response, flagellar biosynthesis and sporulation. Group 4 sigma factors effect extracytoplasmic functions involved in protecting cells from extracellular stresses (PAGET and HELMANN, 2003). Moreover, there is a wide range of sigma factors in different species, e.g. one type sigma factor in *Mycoplasma* sp., seven in *E. coli*, and 63 in *Streptomyces coelicolor*. The more complex the lifestyle of the microorganism is, the more sigma factors can be found in its genome, indicating that the role of sigma factors is important in adaptive capacity (CASES *et al.*, 2003; GRUBER and GROSS, 2003).

RpoS, also known as σ^S in Gram-negative bacteria, belongs to group 2 sigma factors (GROSS *et al.*, 1992). Although it is not essential for bacterial growth, RpoS influences the expression of 10% *E. coli* genes directly or indirectly (LACOUR and LANDINI, 2004; WEBER *et al.*, 2005). The regulator *rpoS* was primarily described in *E. coli* in 1984 (LOEWEN and

TRIGGS, 1984) and then found in other bacterial species (MARTINEZ-GARCIA *et al.*, 2001). In 1991 it was described as a sigma factor-coding gene and named accordingly (LANGE and HENGGE-ARONIS, 1991). The RpoS concentration in cells is controlled by a combination of many signals, including *rpoS* transcription and translation as well as σ^S proteolysis under various stress conditions (HENGGE-ARONIS, 2002; LANGE and HENGGE-ARONIS, 1991; LANGE and HENGGE-ARONIS, 1994; TAKAYANAGI *et al.*, 1994). The half-life of σ^S under non-stress conditions is only one to several minutes, thus RpoS is nearly absent in rapidly growing cells but rapidly and significantly induced when cells are in the early stationary phase (HENGGE-ARONIS, 2002). Moreover, acidic pH, low temperature, and high osmolarity increase the *rpoS* transcription and stimulate the translation of already present *rpoS* messenger RNA (mRNA) (BEARSON *et al.*, 1996; HENGGE-ARONIS, 2002; LANGE and HENGGE-ARONIS, 1994; MUFFLER *et al.*, 1996; TAKAYANAGI *et al.*, 1994). When *rpoS* is repressed in *rpoS*-down or *rpoS*-null mutations, other sigma factors would play an important role in elevating the ability of catabolizing amino acids, e.g. RpoD regulating glucose scavenging and RpoN controlling ammonia assimilation and amino acid uptake, as well as alkaline stress cell protection (FARRELL and FINKEL, 2003).

Another principal sigma factor involved in the stationary phase survival and damage protection in Gram-negative bacteria is RpoH (FREDRIKSSON and NYSTROM, 2006). RpoH is an activator regulating the expression of the heat shock regulon which stimulates the production of chaperones and proteases to repair the oxidative damage caused by the presence of aberrant proteins (FREDRIKSSON *et al.*, 2005).

Shifting the gene expression of sigma factors fine-tunes the relative concentrations of the sigma factors in the cytoplasm, in which competition of different sigma subunits to bind the limited amount of available RNA polymerase core enzymes occurs (ISHIHAMA, 2000). Therefore, the stationary phase is induced by the relative abundance of specific sigma factors (NAVARRO LLORENS *et al.*, 2010). The specific sigma factors involved in the stationary phase are shown in Table 1-4.

Table 1-4: Sigma factors involved in the stationary phase (modified from NAVARRO LLORENS *et al.*, 2010)

Sigma factor	Regulation of	Reference
RpoD (σ^{70})	Fundamental metabolism	YUSTE <i>et al.</i> (2006)
RpoF (σ^{28})	Synthesis of flagella and chemotaxis	YUSTE <i>et al.</i> (2006)
RpoN (σ^{54})	Nitrogen metabolism motility	YUSTE <i>et al.</i> (2006)
RpoH (σ^{32})	Heat shock	YUSTE <i>et al.</i> (2006)
RpoE (σ^{24})	Extra cytoplasmic stress	COSTANZO and ADES (2006)
RpoS (σ^{38})	Stationary phase and stress resistance	YUSTE <i>et al.</i> (2006)
ECF sigma factor	Extracellular functions	MAEDA <i>et al.</i> (2000) YUSTE <i>et al.</i> (2006)

ECF: extracytoplasmic function

The small non-coding RNAs, known as microRNAs and short interfering RNAs (siRNA), have been described as crucial regulatory elements in the regulation of bacterial stress responses (GOTTESMAN, 2005). The length of small non-coding RNAs is usually between 80 to 100 nucleotides (NAVARRO LLORENS *et al.*, 2010). Approximately (approx.) 60 small non-coding RNAs have been reported in *E. coli*, and at least 20 involve in stress responses (GOTTESMAN, 2005; ZHANG *et al.*, 2003). Some small non-coding RNAs require interaction with the bacterial RNA chaperone Hfq at single-stranded AU-rich regions (GOTTESMAN, 2005; VALENTIN-HANSEN *et al.*, 2004). Small non-coding RNAs play a role in adjusting translation and stability of specific target mRNAs by base-pairing with mRNAs (ZHANG *et al.*, 2003). Moreover, they stimulate RpoS translation through pairing an inhibitory stem of a hairpin (HENGGE-ARONIS, 2002). Small non-coding RNAs have also been found modulating the outer membrane proteins (GUILLIER *et al.*, 2006; JOHANSEN *et al.*, 2006). MicA and RybB, two Hfq-binding small RNAs, destabilized the outer membrane protein transcription under induction of RpoE, when cells shifted from rapid growth phase to the stationary phase (JOHANSEN *et al.*, 2006).

Leucine-responsive regulatory protein (Lrp) is a global transcription regulator that can act as either an activator or a repressor depending on the promoter (CALVO and MATTHEWS, 1994; CUI *et al.*, 1996; NEWMAN *et al.*, 1992). Lrp activity, which can be neutral, potentiating, or antagonistic regulated by leucine, seems to harmonize the cellular metabolism with nutritional environment (CALVO and MATTHEWS, 1994; LANDGRAF *et al.*, 1996). Lrp induces the expression of many genes which act in response to nutrient limitation, high concentrations of organic acids, and osmotic stress (TANI *et al.*, 2002). In addition, the integration host factor (IHF), a member of histone-like protein (H-NS) family, promotes the organization of genome. IHF regulates a few of *E. coli* stationary phase genes, e.g. *dps* (ALTUVIA *et al.*, 1994), *osmY* (COLLAND *et al.*, 2000) and curli-producing genes (GERSTEL *et al.*, 2003), and also contributes to the expression of virulence genes in *Salmonella enterica* subsp. *enterica* (*S.*) serovar Typhimurium stationary phase (MANGAN *et al.*, 2006).

Quorum sensing (QS) is a cooperative bacterial behavior that regulates gene expression in response to fluctuations in cell-population density (MILLER and BASSLER, 2001). QS is also used by bacteria for determining the bacterial population density to predict the carrying capacity to prevent population collapse (GOO *et al.*, 2012). When the population density reaches a detection threshold, cells trigger a specific response to approach the stationary phase (KELLER and SURETTE, 2006). Since Goo *et al.* (2012) found that QS mutants lost the cell viability commencing shortly after the onset of the stationary phase, QS might induce important functions involving in the persistence of the stationary phase. QS is also involved in the entry of the stationary phase, as the QS pathways converge with starvation-sensing pathways to regulate the transition of the stationary phase (LAZAZZERA, 2000). QS enables bacteria to modify their physiology in preparation for survival of maximum population at compositive conditions. Moreover, QS gene expression is controlled by RpoS at the onset of the phase (SCHUSTER *et al.*, 2004).

1.4.3.3 The long-term stationary phase

A long-term stationary phase is another strategy for bacteria to maintain long-term survival. The growth rate balances the dying cells in a long-term stationary phase. Long-term cultures can only maintain a certain amount of cells (FINKEL, 2006). A number of bacteria establish a growth advantage in the stationary-phase (GASP) phenotype when are grown in co-culture with the same or other younger bacterial species (HELMUS *et al.*, 2012). The GASP phenotype is a global microorganism phenomenon under starvation conditions (FINKEL *et al.*, 2000), and its purpose is mainly on maximizing long-term total productivity (KEYMER *et al.*, 2008). The GASP phenotype exists in many bacteria, such as *E. coli*, *S.* serotypes, *Shigella dysenteriae* (MARTINEZ-GARCIA *et al.*, 2003), *V. cholerae* (PAUL *et al.*, 2004), *Pseudomonas (P.) aureofaciens* (SILBY *et al.*, 2005) and *Mycobacterium smegmatis* (SMEULDERS *et al.*, 1999).

In summary, the stationary phase is important for bacterial survival against many stresses. Additionally, bacterial cells can survive in the long-term stationary phase without entering the VBNC state or sporulation under starvation periods.

1.5 Viable but non-culturable state

Cultivability is one of the fundamental conditions in microbiology, and the count of microbial colonies on a plate is one of the standard judgments in food hygiene. However, Xu *et al.* (1982) observed a new phenomenon in *E. coli* and *V. cholerae* cells, which was named the viable but non-culturable (VBNC) state. As the name indicated, bacterial cells in this state are alive but have lost the capability of forming colonies on suitable media, on which normal cells can grow (OLIVER, 2000b). In environmental water and soil samples, less than 1% of the microorganisms are cultured in viable count procedures (FAKRUDDIN *et al.*, 2013).

1.5.1 VBNC state bacteria

Since 1982, numerous studies have been conducted to detect the bacteria which enter the VBNC state for survival. The VBNC state existence in disparate environmental or under experimental conditions has been observed in as many as 85 bacterial species (LI *et al.*, 2014). These 85 bacteria species have a wide range of living conditions, including ocean, estuarine, lake, tap, soil, plants and even processed food. The identification of VBNC bacteria has been reviewed and summarized (LI *et al.*, 2014). LI *et al.* (2014) focussed on human bacterial pathogens, Pinto *et al.* (2013) classified pathogenic and non-pathogenic VBNC bacteria, and Oliver (2010) reviewed pathogenic bacteria in plants, animals and human, while Rowan (2004) distinguished foodborne VBNC bacteria from waterborne. There are 67 pathogenic species and 18 non-pathogenic species of in total 85 VBNC species known so far. Of these 67 bacterial species, 51 human pathogenic bacteria are listed in Table 1-5, including species and their VBNC state inducing conditions. This list includes not only pathogens that cause disease or even death, but also opportunistic human pathogenic species that can additionally infect other organisms and immunocompromised patients. The remaining 16 pathogenic species (Table 1-6) have not been described to infect humans but other organisms, e.g. plants, fish, oysters, and corals.

Table 1-5: Human pathogens VBNC state inducing conditions (modified from LI *et al.*, 2014)

Species	VBNC state inducing conditions	References
<i>Acinetobacter calcoaceticus</i>	Low nutrition and cold (winter)	LEMKE and LEFF (2006)
<i>Aeromonas hydrophila</i>	Low salinity (0.35% NaCl)	RAHMAN <i>et al.</i> (2001)
<i>Agrobacterium tumefaciens</i>	Low nutrition (filtered drinking water)	BYRD <i>et al.</i> (1991)
<i>Arcobacter butzleri</i>	Chemical salts form of copper Low nutrition (sterilized seawater microcosms)	ALEXANDER <i>et al.</i> (1999) FERA <i>et al.</i> (2008)
<i>Bacillus cereus</i>	Pulsed electric field (50 Hz, 60V)	ROWAN (2004)
<i>Burkholderia cepacia</i>	Low nutrition and cold (winter)	LEMKE and LEFF (2006)
<i>Burkholderia pseudomallei</i>	Acid pH (pH4) or high temperature (42 °C) or High osmotic pressure (above 2.5% (wt/vol))	INGLIS and SAGRIPANTI (2006)
<i>Campylobacter coli</i>	Low temperature (10 °C)	THOMAS <i>et al.</i> (2002)
<i>Campylobacter jejuni</i>	Low pH (pH4) Low temperature (4 °C or 10 °C)	CHAVEERACH <i>et al.</i> (2003) COOK and BOLSTER (2007); THOMAS <i>et al.</i> (2002)
<i>Campylobacter lari</i>	Low pH (pH4) Low nutrition (sterile artificial seawater microcosm)	CHAVEERACH <i>et al.</i> (2003) BAFFONE <i>et al.</i> (2006b)
<i>Citrobacter freundii</i>	Low temperature (10 °C) Low nutrition (sterile double-distilled water) or high temperature (48 °C or 53 °C)	THOMAS <i>et al.</i> (2002) REISSBRODT <i>et al.</i> (2002)
<i>Cytophaga allerginae</i>	An aerosol with medical-grade air (20 lb/in ²)	HEIDELBERG <i>et al.</i> (1997)
<i>Edwardsiella tarda</i>	In an oligotrophic microcosm and under low temperature (4 °C)	DU <i>et al.</i> (2007)
<i>Enterobacter aerogenes</i>	Low nutrition (filtered drinking water)	BYRD <i>et al.</i> (1991)
<i>Enterobacter agglomerans</i>	Low nutrition (sterile double-distilled water)	REISSBRODT <i>et al.</i> (2002)
<i>Enterobacter cloacae</i>	In sandy loam soil with water stress	PEDERSEN and JACOBSEN (1993)
<i>Enterococcus faecalis</i> (<i>Streptococcus faecalis</i>)	Low nutrition (filtered drinking water) Low temperature (4 °C or 7 °C)	BYRD <i>et al.</i> (1991) LLEO <i>et al.</i> (1998)
<i>Enterococcus faecium</i>	Low nutrition (filtered sterilized lake water) and low temperature (4±0.5 °C)	LLEO <i>et al.</i> (2001)
<i>Enterococcus hirae</i>	Low nutrition (filtered sterilized lake water) and low temperature (4±0.5 °C)	LLEO <i>et al.</i> (2001)
<i>Escherichia coli</i>	Visible light at an intensity of about 40 klux Low nutrition (sterile double-distilled water) or high temperature (53 °C) In deionized water at 4 °C	GOURMELON <i>et al.</i> (1994) REISSBRODT <i>et al.</i> (2002) COOK and BOLSTER (2007)
<i>Francisella tularensis</i>	CO ₂ (5%)	FORSMAN <i>et al.</i> (2000)
<i>Haemophilus influenzae</i>	Unknown	EHRlich <i>et al.</i> (2002)
<i>Helicobacter pylori</i>	Low nutrition (filtered environmental water)	ADAMS <i>et al.</i> (2003)
<i>Klebsiella aerogenes</i>	Unknown inducing condition	OLIVER (2010)
<i>Klebsiella planticola</i>	An aerosol with medical-grade air (20 lb/in ²)	HEIDELBERG <i>et al.</i> (1997)
<i>Klebsiella pneumoniae</i>	Low nutrition (sterile and filtered drinking water)	BYRD <i>et al.</i> (1991)
<i>Legionella pneumophila</i>	Low nutrition (sterile tap water) Add 0.3 g Cl ₂ /L NaOCl to disinfectants NH ₂ Cl	STEINERT <i>et al.</i> (1997) ALLERON <i>et al.</i> (2008)
<i>Listeria monocytogenes</i>	<i>Hartmannella vermiformis</i> supernatant With 0% or 7% NaCl at 20 °C Low pH (pH4)	BUSE <i>et al.</i> (2013) BESNARD <i>et al.</i> (2002) ROWAN (2004)
<i>Micrococcus luteus</i>	Low nutrition (microcosm filtrated water) and low temperature (4 °C) In the filtered supernatant of late logarithmic phase <i>M. luteus</i> cultures	LINDBACK <i>et al.</i> (2010) MUKAMOLOVA <i>et al.</i> (1998b)

<i>Micrococcus varians</i> (<i>Kocuria varians</i>)	Unknown inducing condition	OLIVER (2005)
<i>Mycobacterium smegmatis</i>	In modified Hartman's de Bont medium In Hartman's de Bont medium medium but N-, C- or P-limitation	KUZNETSOV <i>et al.</i> (2004) SHLEEVA <i>et al.</i> (2004)
<i>Mycobacterium tuberculosis</i>	Without oxygen	DOWNING <i>et al.</i> (2005)
<i>Pseudomonas aeruginosa</i>	In sterile deionized water at 4 °C Add copper sulfate (CuSO ₄ × 5H ₂ O)	LEUNG <i>et al.</i> (1995) DWIDJOSISWOJO <i>et al.</i> (2011)
<i>Pseudomonas putida</i>	Low nutrition and cold (winter)	LEMKE and LEFF (2006)
<i>Salmonella</i> ¹ Enteritidis	Low nutrition (sterile river water microcosm)	ROSZAK <i>et al.</i> (1984)
<i>Salmonella</i> ¹ Oranienburg	In 7% NaCl solution	PANUTDAPORN <i>et al.</i> (2006)
<i>Salmonella</i> ¹ Typhi	In groundwater at 15 °C	CHO and KIM (1999)
<i>Salmonella</i> ¹ Typhimurium	In sterilized artificial seawater penetrated by UV-C Low nutrition (sterile double-distilled water) Containing 0.0735 mM Butterfield phosphate solution at 5°C	CARO <i>et al.</i> (1999) REISSBRODT <i>et al.</i> (2002) GUPTA <i>et al.</i> (2003)
<i>Serratia marcescens</i>	An aerosol with medical-grade air (20 lb/in ²)	HEIDELBERG <i>et al.</i> (1997)
<i>Shigella dysenteriae</i>	Low nutrition (sterilized environmental water) High temperature (30 °C or 37 °C)	ISLAM <i>et al.</i> (1993) RAHMAN <i>et al.</i> (1994)
<i>Shigella flexneri</i>	Low nutrition (filtered environmental water)	COLWELL <i>et al.</i> (1985)
<i>Shigella sonnei</i>	Low nutrition (filtered environmental water)	COLWELL <i>et al.</i> (1985)
<i>Staphylococcus aureus</i>	Antibiotic (vancomycin or quinupristin/dalfopristin)	PASQUAROLI <i>et al.</i> (2014)
<i>Staphylococcus epidermidis</i>	Unknown	ZANDRI <i>et al.</i> (2012)
<i>Vibrio alginolyticus</i>	Low temperature (4 °C)	DU <i>et al.</i> (2007)
<i>Vibrio cholerae</i>	Low nutrition (filtered river water)	COLWELL <i>et al.</i> (1985)
<i>Vibrio fluvialis</i>	In sterile seawater plus sediment (1:4, V/V)	AMEL <i>et al.</i> (2008)
<i>Vibrio mimicus</i>	Unknown inducing condition	OLIVER (1995)
<i>Vibrio parahaemolyticus</i>	Low temperature (5 °C), low salinity In a nutrient-free Morita mineral salt-0.5% NaCl medium (pH 7.8) at 4 °C	BATES and OLIVER (2004) WONG and WANG (2004)
<i>Vibrio vulnificus</i> (type 1 & 2)	Low temperature (5 °C) Low salinity (0.4% NaCl)	BIOSCA <i>et al.</i> (1996a) WONG and LIU (2008)
<i>Yersinia pestis</i>	In water at 4 °C	PAWLOWSKI <i>et al.</i> (2011)

¹ *Salmonella*: *Salmonella enterica* subsp. *enterica*

If bacteria inhabit unfavourable environments entering the VBNC state might be forced. Numerous chemical and environmental factors, which may induce VBNC state entering, are described (Table 1-5) (OLIVER, 2010). Factors include loath temperature (BESNARD *et al.*, 2002; MAALEJ *et al.*, 2004; WONG and WANG, 2004), nutrient starvation (COOK and BOLSTER, 2007), low pH (CUNNINGHAM *et al.*, 2009), or low salinity (ASAKURA *et al.*, 2008; WONG and LIU, 2008), high oxygen concentration (KANA *et al.*, 2008), presence of food preservatives (CUNNINGHAM *et al.*, 2009; QUIROS *et al.*, 2009), heavy metals presence/absence (GHEZZI and STECK, 1999) and exposure to white and/or UV light (GOURMELON *et al.*, 1994). However, more extreme conditions may lead to sterilization instead of inducing bacteria cells to enter the VBNC state, e.g. pasteurization.

Table 1-6: Non-human pathogenic bacterial species with a proven VBNC state

Species	Host organisms	References
<i>Aeromonas salmonicida</i>	Fish	EFFENDI and AUSTIN (1995)
<i>Aquaspirillum</i> sp.	Fish	WAI <i>et al.</i> (2000)
<i>Erwinia amylovora</i>	Plant	ORDAX <i>et al.</i> (2009)
<i>Lactobacillus plantarum</i>	Plant	OLIVER (1995)
<i>Pasteurella piscida</i>	Fish	MAGARINOS <i>et al.</i> (1994)
<i>Pseudomonas syringae</i>	Plant	WILSON and LINDOW (1992)
<i>Ralstonia solanacearum</i>	Plant	GREY and STECK (2001)
<i>Rhizobium leguminosarum</i>	Plant	ALEXANDER <i>et al.</i> (1999)
<i>Tenacibaculum</i> sp.	Sea urchins	MASUDA <i>et al.</i> (2004)
<i>Vibrio anguillarum</i>	Fish	EGUCHI <i>et al.</i> (2000)
<i>Vibrio campbellii</i>	Oysters	WILLIAMS <i>et al.</i> (2009)
<i>Vibrio proteolytica</i>	Fish	OLIVER (2005)
<i>Vibrio shiloi</i>	Corals	VATTAKAVEN <i>et al.</i> (2006)
<i>Vibrio tasmaniensis</i>	Corals	VATTAKAVEN <i>et al.</i> (2006)
<i>Xanthomonas axonopodis</i> pv. <i>citri</i>	Plant	DEL CAMPO <i>et al.</i> (2009)
<i>Xanthomonas campestris</i> pv. <i>campestris</i>	Plant	GHEZZI and STECK (1999)

1.5.2 Characteristics of VBNC cells

Viability of bacteria cells means metabolic or physiological activity. However, non-cultivability is one of the very important characters of VBNC cells. Although VBNC cells share several similar viable characteristics, they have many differences from viable and culturable cells. These changes include cellular morphology, composition of cell membrane,

metabolism, expression of genes, resistances, capability of adhesion and virulence. Despite many differences between VBNC cells and culturable cells, VBNC cells are not dead cells. Damaged membranes in dead cells hamper conserve genetic information, whereas VBNC cells maintain intact membranes to protect chromosomes, mRNA and plasmids from damage. No metabolic activity occurs in dead cells, while VBNC cells still maintain metabolism and respiration. Kogure *et al.* (1979) distinguished live cells from dead cells with nalidixic acid (20-40 mg/L) according to metabolism. VBNC cells does not divide and appear elongated after exposure to nalidixic acid (Fig. 1-2), while the nonviable metabolically inactive cells have no change of original shape and size (KOGURE *et al.*, 1979). The production of mRNA and transcription are continued in VBNC cells (LLEO *et al.*, 2000).

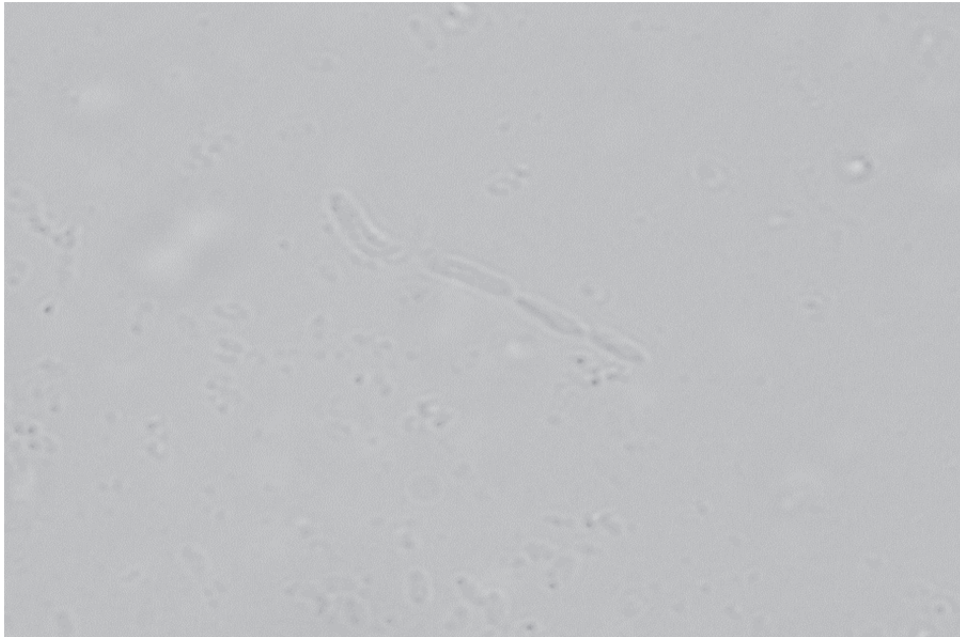


Fig. 1-2: VBNC *V. parahaemolyticus* RIMD 2210633 cells after exposure to nalidixic acid.

The decrease of cell size has been observed in a number of VBNC bacterial species (CHAIYANAN *et al.*, 2001; DU *et al.*, 2007; INGLIS and SAGRIPANTI, 2006; SENOH *et al.*, 2010; THOMAS *et al.*, 2002; WHITESIDES and OLIVER, 1997). The reduction of cell size and an increase in surface area to volume ratio might be a strategy to lessen energy requirements (BIOSCA *et al.*, 1996b; LI *et al.*, 2014). *V. parahaemolyticus* cells change from the rod-shaped in the exponential phase to coccoid in the VBNC state (SU *et al.*, 2013). Studies of morphological changes of *V. cholerae* O1 and O139 cells have found slight differences in detailed changes of shape (CHAIYANAN *et al.*, 2001). *V. cholerae* O1 cells became shorter and fatter, while *V. cholerae* O139 cells came shorter and thinner than actively growing cells at about two months. At six months, both *V. cholerae* O1 and O139 cells were coccoid in morphology when all completely entered the VBNC state. These alterations of cellular morphology could serve as a crucial trait in order to identify the state of VBNC cells. However, it should not be the only factor to judge cells whether they entered the VBNC state because other conditions (e.g. starvation) can lead to similar changes (CHEN *et al.*, 2009).

The change of the cell membrane is another factor which is used to identify the difference between VBNC and normal cells. The outer membrane of Gram-negative bacteria, which is a physical and functional barrier, plays an important role in separating cell from surrounding. *V. cholerae* O1 and O139 VBNC cells were observed under transmission electron microscope by Chaiyanan *et al.* (2001). VBNC cells lost the distinct three-layered integrity of the outer membrane and cell membrane, while the structures residued presented. The nuclear region

was compressed into the cell center surrounded by of a denser cytoplasm. The cell wall formed an extended or convoluted structure pulling away from the cell membrane. Compositions of cell wall and membrane, including fatty acid, peptidoglycan and protein, are investigated. Significant changes in fatty acids composition of *V. vulnificus* cell membranes occurs at the beginning of shifting into the VBNC state (DAY and OLIVER, 2004). The percentage of unsaturated fatty acid and less than 16 carbons fatty acid was found increased in *V. vulnificus* cell membranes during entering the VBNC state. Moreover, hexadecanoic acid (16:0) was found greatly changed in the VBNC state. The peptidoglycan chemical composition of *Enterococcus faecalis* cell wall showed a 9% increase in cross-linked muropeptides values from growing cells to the VBNC cells (SIGNORETTO *et al.*, 2000). However, all these composition changes in VBNC state *Enterococcus faecalis* cells might be specific to this particular physiological state. The outer membrane subproteome of *E. coli* is widely investigated. Using proteomic analysis, changes in protein composition of *E. coli* cells outer membrane subproteome were found when they entered the VBNC state (MUELA *et al.*, 2008). Muela *et al.* (2008) generated VBNC state *E. coli* cells through starvation, incubation in seawater and/or exposure to visible light, and found rearrangements of the outer membrane depending on incubation conditions.

VBNC cells are referred to as dormant cells (COLWELL, 2000), because they show a lower metabolic rate and altered gene expression compared with cells in exponential and stationary phases. The RNA, DNA and protein are decreased under the VBNC state (TREVORS *et al.*, 2012). DNA degradation has been described during cold incubation (WEICHART *et al.*, 1997), and degradation of RNA is reported occurring in populations of starved or stressed bacteria (DAVIS *et al.*, 1986; MANDELSTAM and HALVORSON, 1960; POSTGATE and HUNTER, 1962; WEICHART *et al.*, 1997). Gene expression has been investigated *in vivo* and *in vitro* in *V. vulnificus* VBNC state (FISCHER-LE SAUX *et al.*, 2002; SMITH and OLIVER, 2006a). Previous studies demonstrate that the synthesis of RNA, DNA and protein decreased rapidly in the VBNC state (OLIVER, 2000a). Moreover, expression of *oxyR* and *katG* was not detectable, while *rpoS* was expressed stably after cold shock in *V. vulnificus* (LIMTHAMMAHISORN *et al.*, 2009). Cold shock genes *csp3* and *csp4* underwent an ephemeral induction in VBNC state *V. vulnificus* cells (LIMTHAMMAHISORN *et al.*, 2009). The differences of proteome analysis have been studied between starved and the VBNC state (incubated at 4 ± 0.5 °C for 20 days) *Enterococcus faecalis* cells (HEIM *et al.*, 2002). Considerable changes in protein profiles among exponential phase, starved and VBNC cells were found. GroEL and DnaK, two general stress proteins, showed slight reduction in the VBNC state compared to the exponential phase. Interestingly, three proteins involved in catabolic pathways, the putative protein enolase, ATP synthase and enoyl-ACP reductase, displayed a significant reduction in the VBNC state. Thus, metabolic activity is decreased in the VBNC state. While Lai *et al.* (2009) found that in VBNC cells several proteins associated with e.g. transcription and translation were induced compared with normal cells, which play a role after stimulation and/or maintenance of VBNC state in *V. parahaemolyticus* ST550. However, the elongation factor Tu (EF-Tu) retained the expression level in *E. coli* (ASAKURA *et al.*, 2007b). Furthermore, more than 600 genes with changed expression profiles by more than twofold were found when *V. parahaemolyticus* cells were in cold shock (YANG *et al.*, 2009). WOOD and ARIAS (2011) reported that only a small portion of genes (165 out of 4488) altered their expression considerably (twofold) during the extreme temperature conversion from 35 °C to 4 °C when analyzing the *V. vulnificus* cold shock incitement. However, ASAKURA *et al.* (2007a) has shown that 30 genes in total were expressed stably in the VBNC state, while 21 genes connected to six metabolism-related functional categories (amino acid synthesis; energy metabolism; purines, pyrimidines, nucleosides and nucleotides; central intermediary metabolism; DNA metabolism; as well as protein synthesis) have been induced by at least

fivefold in VBNC cells of *V. cholerae*. GONZALEZ-ESCALONA *et al.* (2006) reported the transcription of key enzymes, such as *tuf* and *relA* or *rpoS*, which are involved in cellular metabolism, is higher in VBNC state *V. cholerae*. These findings suggest that the VBNC state is a physiologically distinct state within the bacterial life cycle, thus its metabolism is changed in terms of expression. The discrepancies of metabolism and gene expression in the VBNC state of different species might be due to the condition inducing the VBNC state.

The VBNC state enables bacteria, especially non-sporulating ones, to survive in hostile environments. Since cell walls might be strengthened by the increase of the cross-linking of peptidoglycans (SIGNORETTO *et al.*, 2000). VBNC cells display higher resistances than normal cells. VBNC state *V. parahaemolyticus* cells were resistant to thermal (42-47 °C), low salinity (0% NaCl), or acidic (pH 4) based inactivation (WONG and WANG, 2004). For *Campylobacter (C.) jejuni* populations, the survival percentages of original rod cells to chlorine (5ppm v/v) for 30s, 2min and 5min were around 15%, 14% and 0%, while the survival percentages of VBNC cells were around 65%, 20% and 8%, respectively (ROWE *et al.*, 1998). *Enterococcus faecalis* VBNC cells were capable of maintaining and expressing antibiotic resistance (LEO *et al.*, 2003). Furthermore, VBNC state *V. vulnificus* was found to exhibit resistance to high temperatures (42 °C), low and high pHs (3 and 10), and exposure to ethanol (13%), antibiotic (chloramphenicol and ampicillin) and heavy metal (zinc) (NOWAKOWSKA and OLIVER, 2013). Weichert and Kjelleberg (1996) found that resistance towards sonication of six-week cold induced VBNC cells were similar to growing cells. Moreover, after sonication for 40 s, the survival percentage of nine-week cold induced VBNC cells (10.2%) was higher than the six-week cold induced VBNC cells (0.15%). The resistance of starved cells to ethanol exposure at the concentration of 10% (v/v) was 100 times higher than growing cells, which might suggest that most cells entered VBNC state (WEICHAERT and KJELLEBERG, 1996).

Invasion of hosts starts with adherence, which represents a crucial step for extracellular bacteria (RIBET and COSSART, 2015). The capability of adhesion in the VBNC state is altered as well. For instance, Pruzzo *et al.* (2002) found that VBNC state *Enterococcus faecalis* cells maintained the adherence capability but its efficiency to attach to cardiac cells was reduced by 40% to 70%. Moreover, VBNC state *Enterococcus faecalis* lost the ability to adhere to plastic surfaces (LEO *et al.*, 2007), though the ability of attachment was retained in VBNC state *C. jejuni* cells (DUFFY and DYKES, 2009).

The ability of bacterial infection under the VBNC state is disputed. A coral pathogen, *V. shiloi*, causes coral death in the VBNC state (BANIN *et al.*, 2000; ROSENBERG and BEN-HAIM, 2002). However, VBNC state *Listeria monocytogenes* cells did not cause infection in immunodeficient mice (CAPPELIER *et al.*, 2005; LINDBACK *et al.*, 2010). Moreover, a gradually time-depending decrease of virulence was shown in VBNC state *V. vulnificus* (OLIVER and BOCKIAN, 1995). The presence of virulence responses of *V. cholerae* in cell cultures suggests that it cholera might be caused under the VBNC state (COLWELL *et al.*, 1996). Moreover toxin genes are expressed in *V. parahaemolyticus* VBNC state (VORA *et al.*, 2005). Furthermore, VBNC state *V. vulnificus* cells express virulence factors (*vvhA*) (FISCHER-LE SAUX *et al.*, 2002). Many studies suggest that the virulence remains in human pathogenic VBNC state bacterial cells but the capability of infection in the VBNC state has been rarely shown. However, under suitable conditions its rapid resuscitation into culturable cells may lead to infection (BAFFONE *et al.*, 2003; DU *et al.*, 2007; OLIVER, 2005; OLIVER and BOCKIAN, 1995).

The characteristics of VBNC state bacterial cells (FAKRUDDIN *et al.*, 2013) include:

- (i) Changing cell shape from rod to coccoid and maintaining apparent cell integrity;
- (ii) Changes in the number and structure of outer membrane proteins;
- (iii) Increased cross-linking of muropeptides in cell wall;
- (iv) Lower metabolic activity;

- (v) Reduced nutrient transport;
- (vi) Continuous gene expression;
- (vii) Possessing cellular activity;
- (viii) Containing a high ATP level and exhibit high membrane potential;
- (ix) Higher resistance to extremely conditions;
- (x) Capability of resuscitation.

1.5.3 VBNC state regulators and effectors

Although many aspects of the VBNC state have been investigated, little is known about the genetic regulation in this state. A wide range of bacterial species exist in the VBNC state. It is suggested that the regulatory mechanisms are very complex. Identifying key regulators of the VBNC state might help to develop detection and control methods for VBNC state pathogenic bacteria in the field of food hygiene.

The involvement of RpoS, a global stress response regulator, in the induction of the VBNC state has been investigated (BOARETTI *et al.*, 2003; KUSUMOTO *et al.*, 2012). A continuous expression of *rpoS* in VBNC state *V. vulnificus* (LIMTHAMMAHISORN *et al.*, 2009; SMITH and OLIVER, 2006a) as well as *V. cholerae* (GONZALEZ-ESCALONA *et al.*, 2006) was reported. A lack of RpoS in *E. coli* and *Salmonella* spp. was shown to result in a faster shift in the VBNC state (KUSUMOTO *et al.*, 2012). The intracellular concentration of RpoS decreased during VBNC state induction in *S. Dublin*, *S. Oranienburg* and *S. Typhimurium* LT2. This suggests that disruption and/or lower concentrations of RpoS could lead a rapid VBNC state induction (KUSUMOTO *et al.*, 2012). *E. coli* parental strains lost their cultivability in 33 days on an artificial oligotrophic medium at 4 °C, while the *rpoS* mutant cells reached the non-culturable state in 21 days (BOARETTI *et al.*, 2003). Furthermore, *rpoS* inactivated in *E. coli* cells resulted in an early death of bacteria cells (BOARETTI *et al.*, 2003).

Stress factors inducing RpoS-mediated stress response have been described, including low temperature (WHITE-ZIEGLER *et al.*, 2008), decreased nutrient availability (MANDEL and SILHAVY, 2005), pH change (CHEVILLE *et al.*, 1996), osmotic stress (HENGGE-ARONIS *et al.*, 1993), and oxidative stress (SAMMARTANO *et al.*, 1986). Depending on the type of stress, RpoS regulated stress response induction includes the expression of guanosine 3',5'-bis(diphosphate) (ppGpp) and guanosine 3'-diphosphate, 5'-triphosphate (p)ppGpp (GENTRY *et al.*, 1993). Deficient (p)ppGpp production leads to a faster VBNC state induction, which is comparable to RpoS mutants; (p)ppGpp is therefore recognized as a positive regulator during the RpoS production (BOARETTI *et al.*, 2003). The intracellular concentration of (p)ppGpp is regulated by RelA and SpoT proteins (MAGNUSSON *et al.*, 2005; POTRYKUS and CASHEL, 2008). In the VBNC state, the expression of *relA* was repressed in *V. cholerae*, followed by (p)ppGpp concentration was modulated down (ASAKURA *et al.*, 2007a). Lower (p)ppGpp concentration might result in a faster induction of the VBNC state.

Presence of reactive oxygen species (ROS) induces the shift into the VBNC state (CUNY *et al.*, 2005). Many studies suggested that ROS are involved in low temperature induced the VBNC state in several bacterial species (BOGOSIAN *et al.*, 2000; KONG *et al.*, 2004; MIZUNOE *et al.*, 1999; WAI *et al.*, 2000). The oxidative stress response regulator OxyR, was first described in *S. Typhimurium* (CHRISTMAN *et al.*, 1985). Kong *et al.* (2004) generated an *oxyR* mutant that lacks catalase activity. This mutant lost its cultivability on solid media at low temperatures as well as at room temperature. The catalase encoded via *katG* in *E. coli* was induced by H₂O₂ and regulated by *oxyR* (MONGKOLSUK and HELMANN, 2002; STORZ and ZHENG, 2000). Moreover, Italiani *et al.* (2011) also constructed an *oxyR* mutant strain, and found that *katG* expression of this mutant strain was decreased. The KatG or catalase activity was not detected in the stationary phase. They conclude that *oxyR* is the main positive regulator of *katG*. In VBNC state *V. vulnificus* cells *katG* was repressed *in vitro* and *in vivo*

(SMITH and OLIVER, 2006a). Thus *oxyR* mutant strain inhibits *katG* expression at any temperature and in any growth phase. Moreover, the indirect lack of catalase results in non-cultivability on routine media, which usually contains hydrogen peroxides (KONG *et al.*, 2004). Therefore, cells might enter into the VBNC state because *oxyR*-mediated catalase activity is inhibited due to low temperature. Recently, an antioxidative enzyme, the alkyl hydroperoxide reductase subunit C (AhpC, AhpC1 and AhpC2), protects cells via its peroxidase activity, by inactivation hydrogen peroxide, peroxy nitrite and organic hydroperoxides (CHAROENLAP *et al.*, 2005; WANG *et al.*, 2013a). Wang *et al.* (2013a) found that both genes, *ahpC1* and *ahpC2*, were protective against ROS, tert-butyl hydroperoxide (t-BOOH), while at 4 °C the protective function of *ahpC2* was higher than that of *ahpC1*. Both *ahpC1* and *ahpC2* genes declined and remained at low concentrations under VBNC state inducing conditions, but only *ahpC2* effected the induction and maintains the VBNC state of *V. parahaemolyticus*. Furthermore, *ahpC1* was protective against H₂O₂ (WANG *et al.*, 2013a). Reduced OxyR functions as a repressor of *ahpC* in cells, whereas oxidized OxyR by H₂O₂ activates *ahpC* expression (CHAROENLAP *et al.*, 2005; LOPRASERT *et al.*, 2000). Thus AhpC might play a key role in regulating the induction of the VBNC state.

As mentioned above, phospholipids, lipopolysaccharides and outer membrane proteins (OMPs) are the main components of the outer membrane (KOEBNIK *et al.*, 2000). In order to survive in stressful conditions, e.g. starvation, or changed osmolarity, the amount of OmpC and OmpF was increased in VBNC state *E. coli* (DARCAN *et al.*, 2009; NIKAIDO and VAARA, 1985; OZKANCA and FLINT, 2002). OmpF and OmpC are the major outer membrane proteins in *E. coli* (MISRA and REEVES, 1987). An *ompC**ompF* double mutant lost its cultivability and entered the VBNC state because this mutant lost the ability to ingest and transport small molecular weight nutrients (DARCAN *et al.*, 2009; OZKANCA and FLINT, 2002). The expression of OmpF and OmpC is regulated by the EnvZ/OmpR system (RUSSO and SILHAVY, 1991). Darcan *et al.* (2009) showed that an *envZ* mutant lost the ability to enter the VBNC state and maintained culturable for a longer time. The inhibition of EnvZ has no significant influence on survival; however, a lack of EnvZ would stop bacteria cells perceiving the changes in surrounding and therefore affects shifting in the VBNC state. Since OMPs play an important role in the induction of the shift into the VBNC state, they are important effectors of the VBNC state.

1.5.4 Detection of VBNC cells

Viability and non-cultivability are the two main characteristics of VBNC cells, thus VBNC cells can be identified via microscope. When cells lose cultivability but the number of viable cells remains high in a sample, these cells are generally considered as VBNC cells. However, some injured cells are unable to grow on the selective media agar with antibiotics or other stressors. Therefore, the first step for detection of VBNC cells should be conducted with non-selective media. Since the injured cells can turn into culturable state on rich medium, they are not considered as VBNC cells even if they own a raised sensitivity to normal growth medium components (PINTO *et al.*, 2013). Hence, establishing the most suitable growth conditions could enable to distinguish injured cells from VBNC cells.

Appropriate detection approaches enable the detection of VBNC cells, which can be then dealt with the suitable methods, thereby the bacterial contamination in samples can be reduced in water and/or food samples. Therefore, it is important to confirm the viability of a VBNC population and detect VBNC cells with suitable techniques. Several aspects, which include cell membrane, metabolism, and gene expression, confirm whether cells enter the VBNC state or are dead. In Table 1-7, common methods for detecting VBNC cells have been listed. These methods can be divided into three groups of types.

First type group is field microscopy methods. Kong *et al.* (1979) described the direct count of viable bacterial cells (DVC). Primary studies on bacterial VBNC state detection relies on the antibiotic sensitivity of bacterial cells. The presence of nalidixic acid, a DNA-gyrase inhibitor, stops cell division. Viable cells are elongated, while dead cells are oval and large (KOGURE *et al.*, 1979). However, this approach is not suitable for Gram-positive and some Gram-negative bacteria (BYRD *et al.*, 1991).

Second type group is fluorescent microscopy methods. *V. cholerae* O1 isolates that tolerate high-level concentrations of nalidixic acid have been found in cholera endemic areas (DAS *et al.*, 2011), and thus with ciprofloxacin is preferable to nalidixic acid. This is because with ciprofloxacin VBNC cells elongate from a coccoid shape to rod-like cells in the absence of cell division. Accordingly, modified fluorescent antibody-direct viable count (DFA-DVC) technique with ciprofloxacin has been performed to detect VBNC state *V. cholerae* (MISHRA *et al.*, 2011). The VBNC state can also be determined by modified fluorescent cell staining procedures. Acridine orange, 4,6-diamino-2-phenyl indole (DAPI), fluorescein isothiocyanate (FITC), indophenyl-nitrophenyl-phenyltetrazolium chloride (INT), and 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) are used as dyes (VILLARINO *et al.*, 2000). The mechanism of acridine orange depends on the ratio of DNA to protein in bacterial cells. Actively reproducing cells at the time of staining will appear green but slow-growing or non-reproducing cells appear orange under microscopy (HOLMBERG *et al.*, 1989). DAPI can be used to display changes in cellular morphology (BESNARD *et al.*, 2000b; DU *et al.*, 2007). FITC is a fluorophore that is extensively used in cell biological studies. It retains the complex pH-dependent fluorescence spectra of fluorescein (MARTIN and LINDQVIST, 1975) and can be covalently linked to the membranes of specific organelles and macromolecules. Living cells appear blue or violet with FITC stains. The activity of electron transport systems is the basement of INT assay (RAHMAN *et al.*, 1994). INT reacts with a dehydrogenase enzyme producing formazone and red color (ZIMMERMANN *et al.*, 1978). Similarly, the Live/Dead BacLight assay, a new differential staining assay, has been recently developed. Two nucleic acid stains, green-fluorescent SYTO 9 stain and red-fluorescent propidium iodide stain. SYTO 9 acts on both live and dead bacteria, whereas propidium iodide only passes through the damaged membranes of dead cells. The propidium iodide stain reduces the activity of SYTO 9 in dead bacteria resulting in a red fluorescent, whereas the live bacteria will stay green fluorescent (STIEFEL *et al.*, 2015). Thus, it enables the counting of total and viable (metabolically active) cells at the same time.

Table 1-7: Methods for the detection of bacterial VBNC cells (modified from RAMAMURTHY *et al.*, 2014)

Method	Indicator	Bacteria	Reference
Antibiotics	Elongation of cells in the presence of nalidixic acid or ciprofloxacin	Gram-negative bacteria	KOGURE <i>et al.</i> (1979); MISHRA <i>et al.</i> (2011)
Acridine orange staining, fluorescent antibody-direct viable count (DFA-DVC)	Active cells appear green	<i>Vibrio cholerae</i> O1	KOGURE <i>et al.</i> (1979); MISHRA <i>et al.</i> (2011)
<i>p</i> -Iodonitrotetrazolium violet assay	Activity based on electron transport system	<i>Shigella dysenteriae</i> type1.	RAHMAN <i>et al.</i> (1994)
CTC and DAPI double staining	Respiratory activity	<i>Campylobacter jejuni</i> , <i>Vibrio parahaemolyticus</i> , and <i>Listeria monocytogenes</i>	BAFFONE <i>et al.</i> (2006a); BAFFONE <i>et al.</i> (2003); CAPPELIER <i>et al.</i> (2005)
Metabolic activity	Accumulation of rhodamine	<i>Francisella tularensis</i>	FORSMAN <i>et al.</i> (2000)
Growth on matrices	Absence of 5-hydroxymethylfuran-2-carboxylic and furan-2-carboxylic acids	<i>Pseudomonas collierea</i>	HARA <i>et al.</i> (2012)
Quantitative PCR	Global expression genes	Vibrios, <i>Escherichia coli</i> , etc.	EILER and BERTILSSON (2006); FRANCO <i>et al.</i> (2012); INOUE <i>et al.</i> (2008); MACHADO and BORDALO (2014); YANEZ <i>et al.</i> (2011)
Quantitative PCR with propidium monoazide	Binding of propidium monoazide with extracellular DNA in dead or membrane-compromised cells inhibits quantitative PCR amplification	<i>Legionella pneumophila</i>	YANEZ <i>et al.</i> (2011)

RT-PCR	Quantification of 16S rRNA	<i>Bifidobacterium</i> spp.	LAHTINEN <i>et al.</i> (2008)
DVC-FISH	Measure of RNA, DNA and mRNA changes	<i>Helicobacter pylori</i>	MORENO <i>et al.</i> (2007)
MALDI-TOF/MS	Identification of differentially expressed proteins	<i>Vibrio harveyi</i> , <i>Enterococcus faecalis</i>	JIA <i>et al.</i> (2013)
Solid phase cytometry and fluorescent viability staining	Identification based on intracellular esterases	<i>Campylobacter jejuni</i>	COOLS <i>et al.</i> (2005)
RING-FISH	Detection of individual genes	<i>Vibrio parahaemolyticus</i>	GRIFFITT <i>et al.</i> (2011)
Biosensor	Detection of beta-d-glucuronidase	<i>Escherichia coli</i>	TOGO <i>et al.</i> (2007)
SELEX technique	DNA aptamer-based viability detection	<i>Salmonella</i> ¹ Typhimurium	LABIB <i>et al.</i> (2012)
Bacteriophages	Lytic activity of live cells	<i>Escherichia coli</i> , <i>Salmonella</i> spp.	BEN <i>et al.</i> (2010); FERNANDES <i>et al.</i> (2014)
Eukaryotic cell extracts	Supplementation with thiosulfate citrate bile salts sucrose agar culture medium (TCBS)	<i>Vibrio cholerae</i> O1	SENOH <i>et al.</i> (2014)

¹ *Salmonella*: *Salmonella enterica* subsp. *enterica*

Third type group is molecular techniques. Ethidium monoazide (EMA) combined with real-time quantitative PCR (qPCR) is able to distinguish dead cells from live cells, including VBNC cells (INOUE *et al.*, 2008). EMA is a DNA intercalating dye that enters bacteria via damaged membranes (NOGVA *et al.*, 2003), thus quantifies DNA selectively from viable cells with intact cell membrane but not dead cells using qPCR (INOUE *et al.*, 2008). RNA is also used for discriminating between culturable and nonculturable cells (FAKRUDDIN *et al.*, 2013). High concentrations of rRNA are maintained in VBNC cells and reductase activity is retained, which are factors essential for all living cells. Thus, quantification of 16S rRNA has been used in several reverse transcription quantitative polymerase chain reaction (RT-qPCR) assays (RAMAMURTHY *et al.*, 2014). Moreover, mRNA is a short-live, half-life less than 1 minute, RNA and only present in metabolically active cells. An mRNA-based RT-qPCR detects non-culturable but alive populations (PAI *et al.*, 2000).

Hybridization probes of nucleic acids and oligonucleotide probes of 18–20 nucleotides are able to discriminate live and dead cells (FAKRUDDIN *et al.*, 2013). Oligonucleotide probes of 18–20 nucleotides rapidly hybridize to specific DNA sequences of target organisms. These probes can detect closely related organisms or organisms with similar functional capabilities (FAKRUDDIN *et al.*, 2013). Hybridization probes of nucleic acids (DNA/RNA) are first labeled with chemically or radioactively markers which detect complementary targets DNA/RNA (JOSEPHSON *et al.*, 1993). Fluorescence in situ hybridization (FISH) is an alternative for hybridization probes. Fluorescently labeled DNA or RNA probes are hybridized to target nucleic acids of intact and permeabilized cells (AMANN *et al.*, 1995; BISHOP, 2010). Single microbial cells have been detected by using rRNA-targeted probes in combination with epifluorescent microscopy. With appropriate rRNA probe sequences, FISH can be applied for detecting all bacterial cells present (a universal probe) or a single population of cells (a strain-specific probe) of e.g. VBNC cells (VILLARINO *et al.*, 2000). Another advanced method, which combined the modified direct viable count method using the DNA-gyrase inhibitor novobiocin with FISH, succeeds in detection of VBNC cells (PIQUERES *et al.*, 2006). Furthermore, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-ToF/MS) with multivariate data analysis is also able to distinguish between resuscitated and non-culturable cells (KUEHL *et al.*, 2011). Different types of blotting, such as colony blot, slot blot, dot blot, and southern blot, can detect VBNC cells directly from the environmental samples (FAKRUDDIN *et al.*, 2013).

1.5.5 Biofilm

Biofilms are defined as structured communities of bacterial cells attached to a surface and enclosed in an exopolysaccharide matrix (MAH and O'TOOLE, 2001; O'TOOLE *et al.*, 2000a). It is produced by single or multiple bacterial species (MAH and O'TOOLE, 2001). Approx. 99% of all existing bacteria produce biofilms (GARRETT *et al.*, 2008). Moreover, it has been estimated that 65% human microbial infections are associated with biofilms (ABDEL-AZIZ and

AERON, 2014). Biofilms are formed under any suitable condition, thus the existence of a surface is perhaps the most important precondition. The first step of biofilm formation includes surface detection (WONG and O'TOOLE, 2011).

At least a part of the biofilm population persists in the VBNC state. For example, the VBNC state of *P. aeruginosa* and *Legionella pneumophila* can be induced by low copper concentrations, indicating that the two species may exist the VBNC state in biofilm in drinking water systems (DWIDJOSISWOJO *et al.*, 2011; WINGENDER and FLEMMING, 2011). *Staphylococcus aureus* lost the cultivability in the biofilm after exposing to antibiotic and/or to nutrient depletion (PASQUAROLI *et al.*, 2013). *C. jejuni* cells shifted into the VBNC state under low nutrient conditions at 4 °C in biofilm (MAGAJNA and SCHRAFT, 2015). Alam *et al.* (2007) reported that biofilm was formed when *V. cholerae* O1 entered the VBNC state. The results indicate that biofilm formation depends on temperature and cultivability hampering conditions. Biofilm protects organisms from nutrient deprivation, pH changes, oxygen radicals, disinfectants, and antibiotics (JEFFERSON, 2004). So far little is known about VBNC cells in biofilm, and the biofilm-associated non-culturable state has not yet been characterized. However, biofilms can still serve as an environmental protector for pathogenic microorganisms and represent an infectious potential to humans.

1.5.6 Resuscitation

Resuscitation was first described by Roszak *et al.* (1984) to explain the recultivability of viable but non-recoverable stage *S. Enteritidis* on solid media with additional nutrients. Resuscitation is defined as the conversion of VBNC cells into culturable cells without any change in cell numbers due to regrowth (BAFFONE *et al.*, 2006a). Until now, 26 of 51 species of human pathogenic organisms, entering the VBNC state, have been reported to resuscitate.

A number of factors influence the success of resuscitation. Many stimuli have been found to play an important role in resuscitating VBNC cells (Table 1-8). Low temperature (4 °C) induced *V. parahaemolyticus* VBNC cells, for example, were successfully resuscitated at 25 °C rather than at 37 °C, indicating that the conditions of resuscitation are crucial (WONG *et al.*, 2004b).

Table 1-8: Stimuli in resuscitating VBNC cells

Stimuli	Bacteria	Reference
Amino acid mixture	<i>Escherichia coli</i>	PINTO <i>et al.</i> (2011)
Gas mixtures	<i>Campylobacter jejuni</i>	BOVILL and MACKEY (1997)
Chicken eggs	<i>Campylobacter jejuni</i> , <i>Campylobacter coli</i>	CHAVEERACH <i>et al.</i> (2003)
ROS	<i>Legionella pneumophila</i>	DUCRET <i>et al.</i> (2014)
Rich media	<i>Campylobacter jejuni</i> , <i>Salmonella</i> ¹ Enteritidis	Cools <i>et al.</i> (2003); ROSZAK <i>et al.</i> (1984)
Rising temperature	<i>Aeromonas hydrophila</i>	MAALEJ <i>et al.</i> (2004)
Supernatants of growing cultures	<i>Escherichia coli</i> , <i>Micrococcus luteus</i>	ARANA <i>et al.</i> (2007); MUKAMOLOVA <i>et al.</i> (1998b)
Tween 20 (3%), catalase (1%)	<i>Salmonella</i> ¹ Typhi	ZENG <i>et al.</i> (2013)

¹ *Salmonella*: *Salmonella enterica* subsp. *enterica*

Incorporation of VBNC cells in natural host animals or chicken eggs have been found to enable resuscitation, such as human volunteers (COLWELL *et al.*, 1996), rabbits (GRIMES and COLWELL, 1986), embryonated eggs of chicken (CHAVEERACH *et al.* 2003), mice (BAFFONE *et al.*, 2006a), clams (BIRBARI *et al.*, 2000), amoebae (GARCIA *et al.*, 2007), and eukaryotic cell lines (SENOH *et al.*, 2010). These successes may be due to the high nutrient concentration in host and the complicated bacteria-host interactions.

Elimination of stress is another influential factor for resuscitating from the VBNC state. Cold temperature and lower nutrition are commonly used for shifting bacterial cells into VBNC state, thus optimal temperature and/or rich medium can help VBNC cells to resuscitate (MUKAMOLOVA *et al.*, 1998b; PINTO *et al.*, 2011; WONG *et al.*, 2004b). Despite the elimination of stressors, a number of specific compounds could also affect the resuscitation of VBNC cells (PINTO *et al.*, 2011). Moreover, autoinducers such as AI-2, RpoS, YeaZ and the resuscitation-promoting factors (Rpfs) also play a role in resuscitation bacterial cells from the

VBNC state (AYDIN *et al.*, 2011; BOARETTI *et al.*, 2003; JOELSSON *et al.*, 2007; LIU *et al.*, 2009; MUKAMOLOVA *et al.*, 1998a). Although resuscitation have been investigated in many bacteria, much more work needs to be done to understand the detailed mechanisms.

1.5.7 VBNC state: a need for further investigation

The VBNC state plays a key role in the survival of a number of non-spore forming bacteria. In case that VBNC cells are present, the total CFU count of bacteria in a sample will be underestimated. Even worse, if all bacteria in the sample are in the VBNC state, the sample may be regarded as a bacteria-free sample due to no detection. As VBNC cells maintain virulence gene expression and can regain virulence after resuscitation, consumption is risky to the hosts including human. Thus, it is important to understand human pathogens that can shift into the VBNC state and then apply reliable detection methods to quantify the precise amount of both culturable and VBNC cells. Apart from this, the identification of the VBNC state inducing conditions as well as the resuscitation conditions are important to effectively prevent bacterial infections and cure infected patients. However, the mechanisms entering and resuscitation of the VBNC state and the methodologies to confirm VBNC bacteria cells need further investigation.

1.6 The aim of thesis

The aim of this thesis was the investigation of the global gene regulation of *V. parahaemolyticus* RIMD 2210633 in three phases of the bacterial life cycle: the phase of exponential growth, the early stationary phase and in the VBNC state. Gene expression profiles were used to analyze differential regulation of genes sharing functional similarities. Moreover, gene enrichment was utilized in order to analyze gene regulation of genes in the same pathways. Up-regulated genes might help to explain how bacterial cells induce entry into the stationary phase and protect them from death and a range of stressors. Genes with stable expression might plot the bottom line of genetic expression important to maintenance of the phase investigated. Accordingly, up-regulated genes were investigated acquiring a better understanding of gene regulation in the both the exponential and stationary phases.

Investigation of *V. parahaemolyticus* VBNC state gene expression might help to understand basic regulation mechanisms in a so called dormant state. Moreover, the development of new detection methods might be supported in order to e.g. avoid foodborne illnesses caused by consumption of seafood containing VBNC state *V. parahaemolyticus* cells.

Chapter 2 Gene expression profiles of *Vibrio parahaemolyticus* in viable but non-culturable (VBNC) state

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Chapter 3 Gene expression profiles of *Vibrio parahaemolyticus* in the early stationary phase

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Chapter 4 Discussions

The viable but non-culturable state is a special state which differs in morphology, physiology and metabolism from the states of culturable cells. Since morphological alteration alone cannot be used as the only factor to judge the VBNC state (PINTO *et al.*, 2013) and there is no prescriptive rule to prove the VBNC state, in this study three different methods have been used to identify the VBNC state.

Ethidium monoazide (EMA) and quantitative PCR (qPCR) were used. EMA is a DNA intercalating dye entering bacteria via damaged membranes (NOGVA *et al.*, 2003). Thus, EMA selectively intercalates in DNA of dead cells. EMA-qPCR is one of the common detecting methods for the VBNC state (JOSEFSEN *et al.*, 2010; QIN *et al.*, 2012). A dynamic range of 4 log₁₀ was obtained for the EMA-qPCR viable/dead detection (RUDI *et al.*, 2005). Another wider detection range for EMA-qPCR (4 log₁₀ with 10 µgmL⁻¹ EMA and 12 log₁₀ with 100 µgmL⁻¹ EMA) was found in *Campylobacter* viable/non-viable cells (SEINIGE *et al.*, 2014). The results in this thesis showed that ΔCt increased from 0.5 to 2.1 while more and more cells entered the VBNC state, which meant that there were more dead cells at day nine than the days before. Finally a minimum of 90% bacterial cells was alive, however, which suggested that in our research the VBNC cells were live cells. Investigations using antibiotics can be used to identify VBNC cells. Nalidixic acid is only effective in Gram-negative bacteria (KOGURE *et al.*, 1979). Enrofloxacin or ciprofloxacin can be used for the same identification VBNC state Gram-positive bacteria as well (BESNARD *et al.*, 2000a). With the suitable amount (100 µg⁻¹) of nalidixic acid VBNC cells in this study appeared elongated (Fig. 1-1), but the non-metabolic active cells showed no change of original shape and size. Thus, nalidixic acid demonstrated that the cells were metabolically active in our study. Additionally, a Live/Dead BacLight staining was performed (data not shown), which is commonly used in recent years (CHAIYANAN *et al.*, 2001; WONG and WANG, 2004). This kit is another method to certify cellular membrane integrity, and it can easily distinguish cells with intact cell membranes from dead cells by color (ROWAN, 2011). Chaiyanan *et al.* (2001) found that 75 to 90% VBNC *V. cholerae* cells were metabolically active. In our study, alive VBNC cells were dominant and retained intact cell membranes, which has the similar to the study of Chaiyanan *et al.* (2001). To sum up, the induction of the VBNC state was successfully applied and the VBNC cells used for RNA extraction were alive and metabolic active cells.

RNA quality plays a crucial role in obtaining reliable results from a microarray gene expression experiment (FASOLD and BINDER, 2014). The RNA Integrity Number (RIN) is referred to as a measure for RNA quality which is determined for most microarray samples before hybridization (SCHROEDER *et al.*, 2006). Moreover, the RIN indexes the degradation of the RNA sample (FASOLD and BINDER, 2014). RIN ranges between 1 and 10, while 7 or greater is recommended for a sample hybridized to a microarray (UPTON *et al.*, 2009). The average RIN of the exponential and the early stationary phases were 9.0 and 7.4, respectively, which were sufficient for a microarray analysis. However, samples with low RNA quality were still valuable for gene expression analysis (OPITZ *et al.*, 2010). Accordingly, although the average RIN value of the VBNC state was 3.3, the samples were still useful. Weichart *et al.* (1997) found a gradual degradation of DNA and RNA in *V. vulnificus* VBNC cells. Similarly, a majority of VBNC cells of *Legionella pneumophila* contained degraded nucleic acid (YAMAMOTO *et al.*, 1996). Therefore, the low RIN value was because the degradation of DNA and RNA, which is a general phenomenon in populations of starved or stressed bacteria and

leads towards the loss of viability (LAHTINEN *et al.*, 2008; LAI *et al.*, 2009; POSTGATE and HUNTER, 1962; WEICHART *et al.*, 1997). Moreover, nine genes, which are crucial for bacterial growth or stress response, were used for confirmed microarray results by RT-qPCR. Similar validations of microarray were performed by Yang *et al.* (2009) and Geurnec *et al.* (2013), with 15 and 18 genes, respectively. The amount of regulation measure by RT-qPCR as well as microarray was comparable in the VBNC state compared with exponential phase or early stationary phase, thus the ratios obtained from microarray were highly correlating (R^2) with expression rates obtained from RT-qPCR (Supplementary material 11-1 and 11-2), indicating that the results of the microarray analyses were reliable.

Comparison of gene expression in the early stationary phase with the exponential phase resulted in 172 up-regulated genes, whereas 61 genes were repressed. In the early stationary phase, up-regulated genes were dominant in five metabolism-related functional categories (central intermediary metabolism; energy metabolism; fatty acid and phospholipid metabolism; biosynthesis of cofactors, prosthetic groups, and carriers as well as protein fate) and five non-metabolic functional categories (cell envelope; transport and binding proteins; regulatory functions; unknown and general functions). In three metabolic functional groups (amino acid biosynthesis; purines, pyrimidines, nucleosides, and nucleotides; and DNA metabolism) no significant up-regulated genes were present (adjusted P -value < 0.05). In total 509 induced genes and 309 repressed genes were found in the VBNC state compared with exponential and early stationary phases. Up-regulated VBNC state genes were dominant over down-regulated genes in two metabolism-related functional categories (fatty acid and phospholipid metabolism; DNA metabolism), while down-regulated genes were dominantly present in five metabolism-related functional categories (amino acid synthesis; central intermediary metabolism; energy metabolism; purines, pyrimidines, nucleosides and nucleotides as well as protein synthesis). Up-regulated genes were regnant in the VBNC state non-metabolism functional categories (transcription; transport and binding). The results confirmed the study of Yang *et al.* (2009), who concluded that genes of non-metabolic functional categories (cell envelope; transport and binding proteins; cellular processes; regulatory functions; mobile and extra chromosomal element functions) as well as unknown function were up-regulated after cold shock.

Regulators and effectors are described in inducing bacterial cells entering the stationary phase and the VBNC state under harmful conditions (BOARETTI *et al.*, 2003; HIRSCH and ELLIOTT, 2005; KUSUMOTO *et al.*, 2012). They are involved in stress response, and they control other genes which might also take part in this response (HENGGE-ARONIS, 2000; WEBER *et al.*, 2005). The following paragraphs discuss the main regulators and effectors.

Sigma factors, a family of transcription factors, play a central role in the transcription of specific subsets of genes/operons and promoter recognition (ISHIHAMA, 2000; TRIPATHI *et al.*, 2014). The number of sigma factors depends on the bacterial species. Until now at least five sigma factors, VP0404 (*rpoD*); VP2358 (*rpoD*); VP2553 (*rpoS*); VP2670 (*rpoN*); and VP2953 (*rpoH*), and six putative sigma factors, VP0055 (the extracytoplasmic function factors, ECF); VP2210 (*rpoD*); VP2232 (*rpoF*); VP2578 (*rpoE*); VPA1555 (*rpoF*); and VPA1690 (*rpoD*), have been found in *V. parahaemolyticus* (WHITAKER *et al.*, 2010). Of these, the RpoS is the most crucial sigma factor in survival under various stressful conditions including starvation, extreme temperature, low pH, oxidative stress, osmotic shock, and exposure to UV light (DNA damage) (BADGER and MILLER, 1995; FANG *et al.*, 1992; LANGE and HENGGE-ARONIS, 1991; LIN *et al.*, 2002; RAMOS-GONZALEZ and MOLIN, 1998; ROSCHE *et al.*, 2005; SUH *et al.*, 1999; TIAN *et al.*, 2008; YILDIZ and SCHOOLNIK, 1998). Therefore it is important for stationary phase and the VBNC state. *rpoS* is reported to induce in the stationary phase (HENGGE-ARONIS *et al.*, 1993). However, VP2553 (*rpoS*) was induced approx. 3 times in the early stationary phase compared with the exponential phase. Since our

definition was that the significant changed gene expression should be over 4 times or below 4 times, VP2553 was considered as stable expression in our research. However, an upward tendency was shown. Because the time point of RNA extraction for our research was the beginning of the early stationary phase, the expression of *rpoS* was not significantly induced. Moreover, our results confirmed the findings of Coutard *et al.* (2005) and Smith and Oliver (2006a), which reported a continued *rpoS* expression in the VBNC state of *V. vulnificus* and *V. parahaemolyticus* Vp4 cells. RpoD, also known as σ^{70} , regulates fundamental metabolic genes which are necessary for cellular growth and survival (WATSON *et al.*, 1998; YUSTE *et al.*, 2006). Klancnik *et al.* (2006) reported that no induction of *rpoD* expression was noticed when cells were starving. Our results suggested that VP2210 (*rpoD*) was significantly down-regulated more than 7 times in the VBNC state compared with that in exponential and early stationary phases (adjusted *P*-value = 8×10^{-5} and 2×10^{-4}), and the VP2358 (*rpoD*) and VPA1690 (*rpoD*) showed no expression change in the early stationary phase and the VBNC state.

In *E. coli* RpoE has been reported playing a role in sensing extra extracytoplasmic stresses including heat shock, ethanol stress, and misfolded periplasmic or outer membrane proteins (DE LAS PENAS *et al.*, 1997; MECSAS *et al.*, 1993; MISSIAKAS *et al.*, 1996; ROUVIERE and GROSS, 1996; WALSH *et al.*, 2003). Compared to the wild type a lower survival rate of an *rpoE* deletion mutant were found after bacterial cells in polymyxin B, ethanol, and high temperature stresses (HAINES-MENGES *et al.*, 2014). The *rpoH* gene positively regulates the transcription of heat shock genes in *E. coli* (ERICKSON and GROSS, 1989; NAGAI *et al.*, 1990; WANG and KAGUNI, 1989). The *rpoH* gene positively regulates the transcription of heat shock genes in *E. coli* (VERSTEEG *et al.*, 2003). Both, *rpoE* and *rpoH* constitutively expressed in cold stress induced VBNC *V. cholerae* cells (ASAKURA *et al.*, 2007a). In our study, VP2578 (*rpoE*) did not show any expressional changes in the early stationary phase compared with the exponential phase, but expression induces approx. 3 times in the VBNC state. Since our definition was that the significant induced gene expression should be above 4 times, VP2578 was considered as stable expression. However, the tendency was shown toward up-regulated. Moreover, no expression changes of VP2953 (*rpoH*) in the early stationary phase and the VBNC state were observed. RpoF governs transcription of the genes for flagella and chemotaxis (ARNOSTI and CHAMBERLIN, 1989; OHNISHI *et al.*, 1990). The concentration of RpoF was found staying constant from the middle exponential phase to the stationary phase (JISHAGE *et al.*, 1996). Our results confirmed the stable RpoF concentration, since genetic expression was measured and found the expression of VP2232 (*rpoF*) and VPA1555 (*rpoF*) was stable in the early stationary and in the VBNC state.

RpoN, a global regulator, controls flagella synthesis as well as playing a crucial role in nitrogen utilization regulation. *rpoN* was found induced in the stationary phase (SUN *et al.*, 2013), however, VP2670 (*rpoN*) was induced approx. 3 times in our study in early stationary phase compared with exponential phase. Since the definition in our research was that the significant induced gene expression should be over 4 times, VP2670 was considered as stable expression. However, the tender was on the rise. The time point of RNA extraction for our research was the beginning of the early stationary phase; therefore the expression of *rpoN* was not significantly induced. The significant down-regulated (approx. 4 and 12 times) in the VBNC state compared with exponential and early stationary phases (adjusted *P*-value = 1×10^{-4} and 4×10^{-8}) were found. Significant reduction of *rpoN* expression demonstrated that there was no requirement of motility for VBNC cells and no need for glutamine syntheses in starvation induced VBNC cells. Moreover, an *rpoN* deletion mutant was found non-motile, and this deletion caused a reduction of biofilm formation and a reduced glutamine synthetase production (WHITAKER *et al.*, 2014). In the early stationary phase and the VBNC state, some

sigma factors with altered expression adjust the related genes, and accordingly, help the bacterial cells to survive in stress conditions.

RpoS is positively regulated by the intracellular accumulation of two small molecules guanosine 3', 5'-bis (diphosphate) (ppGpp) and guanosine 3'-diphosphate 5'-triphosphate (pppGpp), collectively termed (p)ppGpp (BOARETTI *et al.*, 2003; GENTRY *et al.*, 1993). It has been found that the concentration of (p) ppGpp is elevated during stressful conditions and results in an increase in the amount of RpoS (GENTRY *et al.*, 1993). In stressed cells elevated intracellular (p)ppGpp concentration induces gene transcription for stationary phase survival (CHOY, 2000; STEPHENS *et al.*, 1975), and inhibits the transcription of rRNA, tRNA, protein synthesis, and DNA replication (CASHEL *et al.*, 1996; MILON *et al.*, 2006; SVITIL *et al.*, 1993; WANG *et al.*, 2007).

Accumulation of (p)ppGpp depends on the activity of RelA and SpoT (MAGNUSSON *et al.*, 2005; POTRYKUS and CASHEL, 2008). RelA is able to synthesize (p)ppGpp only in case of amino acid starvation (DAS *et al.*, 2009), while SpoT activates (p)ppGpp synthesis under glucose starvation (XIAO *et al.*, 1991) or fatty acid starvation (BATESTI and BOUVERET, 2006; SEYFZADEH *et al.*, 1993) or iron depletion (VINELLA *et al.*, 2005). In this study, the expression of VP2564 (*relA*) and VP0159 (*spoT*) in early stationary phase remained stable compared to the exponential phase, suggesting that no induction of the (p)ppGpp synthesis occurred in the early stationary phase. Therefore, we hypothesized that in our research no starvation of amino acid, glucose, fatty acid and iron in the early stationary phase. While the *rpoS* was induced approx. 3 times, suggesting that (p)ppGpp is referred to as a positive regulator for RpoS but not the only one. In the VBNC state, however, the expression of VP2564 and VP0159 are different. In *V. cholera* VBNC cells the expression of *relA* was repressed, followed by the down-regulated (p)ppGpp concentration (ASAKURA *et al.*, 2007a). However, Nowakowska and Oliver (2013) reported that there was no alteration in the expression of *relA* and *spoT* between VBNC and logarithmic cells of clinical *V. vulnificus* C7184/k2. In our study, the expression of *relA* was repressed approx. 3 times in VBNC state *V. parahaemolyticus* cells, but *spoT* was significantly induced approx. 5 times in the VBNC state compared with exponential and early stationary phases (adjusted *P*-value = 3×10^{-5} and 5×10^{-7}).

VP3003 (*gppA*), a guanosine pentaphosphate phosphohydrolase which converses pppGpp to ppGpp, showed no difference between VBNC cells and exponential and stationary cells in this study. In our study VP2564 was down-regulated in the VBNC state, which indicated that no amino acid starvation occurred. The up-regulation of *spoT* suggested that (p)ppGpp might be synthesized in these VBNC cells under the condition of glucose and/or fatty acid starvation, or iron depletion, while (p)ppGpp is hydrolysed. Because fatty acid and phospholipid metabolism up-regulated genes were dominant in the VBNC state and the VBNC state induced condition mainly depended upon cold temperature, we speculated that (p)ppGpp was hydrolysed in the VBNC state. Therefore, (p)ppGpp might effect RpoS regulation and also plays an important role in cell survival under cold stress conditions.

Reactive oxygen species (ROS), which production is one of the early responses of host instinctive immunity, are yielded to deal with microbial invaders. ROS is a collective term that broadly describes free oxygen radicals which are highly toxic to pathogens and affect the growth of microorganisms (CIRCU and AW, 2010). ROS inactivate bacteria through reacting with vital molecular components of cells, such as DNA, membrane lipids and other cellular components, resulting in the delay of bacterial growth and subsequent death (CADET *et al.*, 2005; KAWANISHI and HIRAKU, 2001; PIZARRO and ORCE, 1988). The concentration of ROS is kept at a tolerable steady state which is balanced by not only the interactions of superoxide dismutases and reductases but also the interactions of peroxidases and catalases (IMLAY, 2008). Moreover, ROS induces the VBNC state via causing oxidative damages on the intracellular macromolecules (CUNY *et al.*, 2005). Many studies have shown that ROS is

involved in the induction of the VBNC state due to low temperature in *V. vulnificus* and other bacterial species (BOGOSIAN *et al.*, 2000; KONG *et al.*, 2004; MIZUNOE *et al.*, 1999; WAI *et al.*, 2000).

OxyR, an oxidative stress response regulator (CHRISTMAN *et al.*, 1985) is activated by intracellular H₂O₂ stress, and directly stimulates synthesis of related proteins such as *yaiA*, *yljA*, and *ybjM* (ZHENG *et al.*, 2001). OxyR-mediated catalase activity digests H₂O₂ present in the media (KONG *et al.*, 2004). Kong *et al.* (2004) also concluded that *V. vulnificus* cells entered the VBNC state because OxyR-mediated catalase activity was blocked by low temperature, which resulted in a raised sensitivity to H₂O₂. Limthammahisorn *et al.* (2009) extracted *V. vulnificus* RNA from oysters which were incubated at 35 °C and 15 °C followed by a 4 °C incubation, at various time intervals from 0 h to 168 h. However, *oxyR* expression was not detected at any time point. However, in our study VP2752 (*oxyR*) was significantly induced in *V. parahaemolyticus* VBNC cells compared with exponential and early stationary cells (adjusted *P*-value = 6×10^{-7} and 2×10^{-9}). Two phenomena might explain this. First, ROS might be naturally present in the media and the up-regulation of *oxyR* induces the OxyR-mediated catalase activity to keep bacterial VBNC cells alive. Second, the up-regulation of *oxyR* was not caused by ROS but by other signals, e.g. indole (VEGA *et al.*, 2012). Since enriched cultured media have been described to produce the ROS upon exposure of the media to daylight and atmospheric oxygen (GRZELAK *et al.*, 2001; VARGHESE *et al.*, 2012), it can be speculated that the up-regulation of *oxyR* in our research was because of present ROS.

KatG, one of the genes encoding the periplasmic catalase of *E. coli* and *V. vulnificus*, is induced by H₂O₂ and positively regulated by *oxyR* (MONGKOLSUK and HELMANN, 2002; SMITH and OLIVER, 2006b; STORZ and ZHENG, 2000). The expression of *katG* was down-regulated *in vitro* and *in vivo* of *V. vulnificus* VBNC state (SMITH and OLIVER, 2006a) as well as in *V. vulnificus* VBNC cells (day 70) compared to day zero cells in artificial sea water (RAO *et al.*, 2014). Both VPA0453 and VPA0768 encode *katG* in *V. parahaemolyticus*. VPA0453 was significantly induced (approx. 8 and 4 times) in the VBNC state (adjusted *P*-value = 2×10^{-8} and 4×10^{-6}), whereas VPA0768 was not altered in the VBNC state compared with in exponential phase but significantly down-regulated (approx. 12 times) in the VBNC state compared with the early stationary (adjusted *P*-value = 9×10^{-9}). Because the positive regulator *oxyR* was up-regulated in the VBNC state in our study, induced expression of *katG* was expected and confirmed by our results.

The alkyl hydroperoxide reductase subunit C (AhpC), which is also regulated by OxyR, is responsible for the detoxification of ROS (CHAROENLAP *et al.*, 2005; WANG *et al.*, 2013a). AhpC is encoded by two *aphC* gene loci on both chromosomes of *V. parahaemolyticus* RIMD2210633, the VP0580 on chromosome 1 (*ahpC2*) and VPA1683 (*ahpC1*) on chromosome 2 (LAI *et al.*, 2009; WANG *et al.*, 2013a). Wang *et al.* (2013a) reported a rapid reduction of expression of both *ahpC* genes during the beginning of the induction of the VBNC state and a remaining low expression in the VBNC state. However, in our research VP0580 (*ahpC2*) was significantly repressed (approx. 12 and 9 times) in *V. parahaemolyticus* VBNC cells, while VPA1683 (*ahpC1*) showed no difference. This result is consistent with the study of Lai *et al.* (2009), who reported that one AhpC homologue quantity was not significantly different in the VBNC induction from that in the VBNC cells and another homologue was markedly down-regulated. Although many studies have suggested that ROS and OxyR play an important role in the induction and maintenance of the VBNC state, the mechanism is not clear yet, therefore further investigation is needed to better understand the regulation.

Unlike RpoS, (p)ppGpp, ROS and OxyR, the following three regulators have been reported to affect mainly the stationary phase gene expression. The first one is Hfq, a global regulator

present in a wide range of bacteria (CHAO and VOGEL, 2010). Hfq interacts with numerous regulatory small non-coding RNAs (sRNAs), e.g. 15-46 known sRNAs in *E. coli* (ZHANG *et al.*, 2003). sRNAs are referred to as important regulatory elements in various biological processes (MASSE *et al.*, 2003; STORZ, 2002). Hfq might directly or indirectly impact the expression of at least 20% of all genes in *S. serotypes* (ANSONG *et al.*, 2009; SITTKA *et al.*, 2008). Thus, this protein affects cellular functions, e.g. stress response, gene expression regulation, and bacterial pathogenicity as well as anti-oxidative functions (BROWN and ELLIOTT, 1996; DING *et al.*, 2004; SU *et al.*, 2010; VYTVYTSKA *et al.*, 1998). Hfq regulates gene expression for survival under various harmful conditions, such as acid, high temperature, osmotic and oxidative stress conditions (MUFFLER *et al.*, 1997; TSUI *et al.*, 1994). Christiansen *et al.* (2004) found Hfq was necessary for the resistance to ethanol and salt but not the oxidative and acid stress in *Listeria monocytogenes*. The transcription of *hfq* was induced at the entry into the stationary phase. In our study, stable expression was found for VP2817 (*hfq*) in the early stationary phase. As *hfq* is regulated by RpoS and the expression of *rpoS* was not induced in the early stationary phase, *hfq* expression might have not been induced yet.

The second regulator is a global transcription regulator the leucine-responsive regulatory protein (Lrp) (CALVO and MATTHEWS, 1994; CUI *et al.*, 1996; NEWMAN *et al.*, 1992). Lrp regulates the expression of genes induced in the stationary phase, which act in response to nutrient limitation, high concentrations of organic acids, and osmotic stresses (TANI *et al.*, 2002). The expression of the *lrp* promoter is repressed in rich medium (CALVO and MATTHEWS, 1994). Moreover, *lrp* expression levels are also decreased during the lag phase in a rich medium, remained steady during exponential phase, and then went back to the starting levels upon entering the stationary phase (LANDGRAF *et al.*, 1996). The results in our research confirmed the previous study since the expression of VP1104 (*lrp*) was stable in the early stationary phase compared with exponential phase.

The third one is a global gene regulator the histone-like protein (H-NS). The H-NS is a major component of the bacterial nucleoid and takes part in promoting the structure and function of chromosomal DNA (ATLUNG and INGMER, 1997; WILLIAMS and RIMSKY, 1997). Moreover, H-NS inhibits the expression of *rpoS* and regulates stationary phase genes (e.g. *osmY*) in *E. coli* (ALTUVIA *et al.*, 1994; BARTH *et al.*, 1995; COLLAND *et al.*, 2000; GERSTEL *et al.*, 2003). Expression of *hns* is necessary for survival during early stationary phase (CHIB and MAHADEVAN, 2012), thus *hns* was induced in the stationary phase in *E. coli* (DERSCH *et al.*, 1993). In our study, however, VP1133 (*hns*) showed no altered expression during the early stationary phase. We speculated that because the point for RNA extraction was the beginning of the early stationary phase, no induction of VP1133 was observed. All these regulators showed different changes of expression in the VBNC state and early stationary phase, indicating that they play different roles in induction and maintenance of the survival.

Since the synthesis of proteins, RNA and DNA decreases in the VBNC state, metabolic activities are reduced (OLIVER, 2000a). During the nutrient starvation, carbohydrates are catabolized first, and then followed by proteins and some RNAs, while DNA is generally but not always protected (TREVORS, 2011). Hence, to investigate the changes of metabolic activity can have a better understand of the bacterial survival mechanism in the early stationary phase and the VBNC state.

In order to adapt to stress conditions, bacteria developed mechanisms which support survival in these changes. The induction of specific carbohydrate transporters is involved in the survival mechanisms. In *V. parahaemolyticus* glucose ingestion is realized via two systems: a carbohydrate phosphotransferase system (PTS) and a sodium-coupled permease (KUBOTA *et al.*, 1979; SARKER *et al.*, 1997; SARKER *et al.*, 1994). Genes involved in the PTS system, including VP0710, VP2046, VPA0298, VPA1421 and VPA1422, were down-

regulated more than 4 times in the early stationary phase. However, VP2636 and VPA0811 were induced more than 4 times in the VBNC state, while VPA0229 was down-regulated more than 13 times. VP0710 and VP2046 were repressed in the VBNC state compared with exponential phase, whereas VPA0298 and VPA1422 were up-regulated in the VBNC state compared with the early stationary phase. Almost all genes involved in the PTS system were down-regulated in the early stationary phase, suggesting that the glucose concentration in the media was absent and cells did not utilize the glucose as carbon. The alteration of gene expression in the PTS system indicated that VBNC cells were using other carbon sources but glucose.

The sodium-coupled permease system depends on Na^+ which plays a role in transporting various nutrients. Na^+ as well as H^+ are coupling cations in the energy transduction in *V. parahaemolyticus* (OKABE *et al.*, 1991; SAKAI *et al.*, 1989). The Na^+/H^+ antiporter activity results in an electrochemical potential establishment of Na^+ and acts as the driving force for nutrient transport (KURODA *et al.*, 1994). VP0618, VP1134, VP1229 and VP2119, which are Na^+/H^+ antiporter genes, showed stable expression in the early stationary phase compared with the exponential phase. In our study, however, significant inductions of VP0618 (approx. 5 and 14 times), VP1134 (approx. 4 and 10 times) and VP2119 (approx. 8 and 14 times) in the VBNC state were found, while only VP1229 was repressed (approx. 4 and 7 times) significantly. These findings suggested that the expression of genes supporting glucose intake had been changed in *V. parahaemolyticus* VBNC cells.

The response to carbon source availability in general seems also to be of special importance, since a replacement of severed carbohydrates was found in bacteria (BOUR, 2012). Bour (2012) studied the altered metabolic profiles in *E. coli* and *S. Typhimurium*, including the loss of the production of carbohydrates, such as glucose, mannose, melibiose, rhamnose, sorbitol and sucrose. Trevors (2011) found the total carbohydrate concentration is lower in the cytoplasm during starvation survival. In our study, the change in carbohydrate composition from using glucose to glutamate has been found. Since *V. parahaemolyticus* is able to use glutamate as single carbon source (ONGAGNA-YHOMBI and BOYD, 2013), the expression increase of glutamine synthesis suggests the alteration of carbohydrate composition. VP0481 (*gltD*) and VP0482 (*gltB*), were significantly induced (approx. 13 and 9 times; 9 and 10 times) in the VBNC state. Meanwhile, VPA0031 (*gltS*) was up-regulated in the VBNC state. However, glutamine synthetase genes, VP0121 (*glnA*), VP0423 (*glnE*), VP0617 (*guaA*), VP1779 and VP1781 were not induced. Since some glutamine synthetase genes were induced, we speculated that glutamate is synthesized in the VBNC state and then consumed as carbon source. Glutamate synthesis is a specific carbohydrate utilization system, which depends on the existence of the corresponding carbon source. This system would be repressed when a more efficiently utilizable carbohydrate is present.

Another enzyme the fructose-bisphosphate aldolase plays an important role in the central metabolic pathway of carbohydrate, e.g. carbon fixation, fructose metabolism, gluconeogenesis, glycolysis, mannose metabolism and the pentose phosphate pathway (HEIM *et al.*, 2002). Fructose-bisphosphate aldolase is activated when bacteria are grown under nitrogen-limiting conditions (SCHMI *et al.*, 2000). The fructose-bisphosphate aldolase was found overexpressed in *Streptococcus pneumonia* VBNC cells compared with exponential growth phase cells but down-regulated in starved cells (HEIM *et al.*, 2002). In our study, VP2599 (*fba*) was significantly repressed approx. 6 times in the *V. parahaemolyticus* VBNC cells compared with exponential growth cells, whereas the expression of VP2599 was approx. 2 times repressed in the VBNC state compared with early stationary phase. The down-regulation of VP2599 might suggest that the carbohydrate metabolic pathway via fructose-bisphosphate aldolase is repressed in the VBNC state. To sum up, some of the genes involved in the carbohydrate transport system and the specific carbohydrate utilization system altered

the expression, suggesting a change in carbohydrate metabolism, such as utilizing glutamine instead of glucose as carbon source.

The nitrogen metabolism is as important as the carbohydrate metabolism. RpoN is a widely distributed sigma factor which regulates nitrogen metabolism (WOLFE *et al.*, 2004). A *V. parahaemolyticus* *rpoN* mutant was found lose the ability to synthesize glutamine synthetase (WHITAKER *et al.*, 2014). RpoN-independent transcription of glutamine synthetase is common in *Vibrio* spp. (KAWAGISHI *et al.*, 1997; KLOSE and MEKALANOS, 1998; WHITAKER *et al.*, 2014; WOLFE *et al.*, 2004). In the early stationary phase, VP0118 (*ntrC*) and VP0119 (*glnL*) showed stable expression. The ratio of VP2670 (*rpoN*) was induced approx. 3 times, suggesting that the nitrogen metabolism might be up-regulated in expression. However, *rpoN* expression was significantly reduced (approx. 4 and 12 times) in the *V. parahaemolyticus* VBNC cells, indicating that the nitrogen metabolism might be reduced. However, there was no difference of the expression of the two nitrogen regulation protein genes, *ntrC* and *glnL*, in the VBNC state. VBNC cells consume nitrogen sources (e.g. NH_4^+) when available. Zeng *et al.* (2013) found that the content of $(\text{NH}_4)_2\text{SO}_4$, which is used as nitrogen source, declined in the medium with *S. Typhi* VBNC cells, but this decline is slower than incubating normal cells. The reduced nitrogen metabolism in the VBNC state confirmed the study of Zeng *et al.* (2013), suggesting that the VBNC cells remained viable.

Moreover, glutamine synthetase regulates the expression of the genes that are involved in nitrogen metabolism (PAHEL *et al.*, 1978). Pahel *et al.* (1978) reported an *E. coli* mutant (*gltB*) lacking glutamate synthase was unable to utilize many compounds as exclusive nitrogen source (e.g. arginine, proline, γ -amino butyrate and glycine). The molarity of two amino acids, glutamate and glutamine, increased in the presence of osmotic shock (NICOLÒ and GUGLIELMINO, 2012). In the early stationary phase, glutamine synthetase genes, VP0617 (*guaA*) and VP1779 were significantly reduced approx. 9 and 5 times, respectively (adjusted *P*-value < 0.05). However, other glutamine synthetase genes, VP0121 (*glnA*), VP0423 (*glnE*), VP0481 (*gltD*), VP0482 (*gltB*), VP1781 and VPA0031 (*gltS*) showed no significant differences in expression. Moreover, VP0481, VP0482 and VPA0031 were significantly up-regulated approx. 13 and 9 times; 9 and 10 times; 7 and 5 times in VBNC cells. However, glutamine synthetase genes, VP0121, VP0423, VP0617, VP1779 and VP1781 were not induced.

Nitrite reductase is an enzyme which reduces nitrite to nitric oxide (NO). The expression of nitrite reductase only happens under the low oxygen conditions, which have been found to reduce culturability in bacterial cells, suggesting that it is referred to as a strategy to induce the VBNC state (CASELLA *et al.*, 2006). Moreover, NirB, NirD, NrfA and NrfC constitute the nitrite reductase. In this study, VP1927 (*nrfC*) and VP1929 (*nrfA*) were significantly repressed around 9 times in the VBNC state compared with exponential phase but displayed expression only 3 times down-regulation in the VBNC state compared with the early stationary phase. VPA0986 (*nirD*) and VPA0987 (*nirB*) were down-regulated as well 3 times in the VBNC state. VPA1054 (*nirD*) showed no difference in the VBNC state, but VPA1055 was significant up-regulated approx. 9 times in the VBNC state compared with stationary phase. The change in the expression of all these nitrite reductase related genes indicates that in both the early stationary phase and the VBNC state microtoxic conditions were absent. Although nitrogen metabolism might be repressed in the VBNC state, an increase of glutamate synthase would enable bacterial cells to utilize a wide variety of nitrogen sources and therefore supports bacterial survival.

The alteration of expression of genes of the fatty acid metabolism occurs when environmental temperature decreases. The fatty acid composition changes of membranes were observed in *V. parahaemolyticus* cells, which demonstrated an increase in the ratio of saturated/unsaturated as well as the concentration of C15:0 fatty acids but a decrease in C16:1

(WONG *et al.*, 2004a). In contrast, the composition changes of membrane fatty acids were also observed in *V. vulnificus* cells, which showed an increased concentration C16:1 during entry into the VBNC state (DAY and OLIVER, 2004). Fatty acids maintain an optimum membrane fluidity to enter and survive in the VBNC state. The inhibition of fatty acid synthesis in bacterial cells can cause cellular death which suggests fatty acid metabolism is essential for bacteria entering the cold induced VBNC state. Saturated fatty acids are converted into unsaturated fatty acids by the enzymes desaturases, while the synthesis of short-chain fatty acids to long-chain fatty acids, branched-chain fatty acids to straight-chain fatty acids, and anteiso-fatty acids to iso-fatty acids are preferential at low temperature (SUUTARI and LAAKSO, 1994). A number of genes, which encode enzymes involved in the degradation of the branched-chain amino acids, were transcriptionally up-regulated via a temperature downshift from 37 °C to 18 °C (KAAN *et al.*, 2002).

Cells synthesize branched-chain fatty acids by utilizing the intermediates of isoleucine and valine degradation (α -methylbutyryl-CoA and isobutyryl-CoA) as substrates. This degradation of isoleucine and valine is mediated by the *mmsA* encoded enzyme methylmalonate semialdehyde dehydrogenase (SABIROVA *et al.*, 2008). Koburger *et al.* (2005) found that the propionyl-CoA corresponding gene *mmsA* was induced about twofold in the stationary phase. They also concluded that the propionyl-CoA was involved in the methyl citrate pathway and was produced by degradation of the branched-chain fatty acids in glucose starved *Bacillus subtilis* cells. *mmsB*, which encodes 3-hydroxyisobutyrate dehydrogenase, also takes part in isoleucine and valine degradation (STEELE *et al.*, 1992). In this study, in the early stationary phase VPA1118 (*mmsB*) and VPA1122 (*mmsA*) were both significantly induced by more than 11 times (adjusted *P*-value < 0.005). In the VBNC cells compared with exponential growth cells VPA1118 and VPA1122 were significantly induced more than 6 times, whereas compared with early stationary phase, VPA1118 showed no difference but VPA1122 demonstrated down-regulated expression in the VBNC state. Moreover, VP2517 (*lpdA*), VP3060 (*ilvE*) and VP3061 (*ilvD*) also play a role in the degradation of isoleucine and valine. VP2517 retained stable in both the VBNC state and the early stationary phase. No expression changes of VP3060 and VP3061 were observed in the early stationary phase. However, VP3060 and VP3061 were significantly down-regulated more than 6 times in the VBNC state. These findings indicate that only some intermediates of isoleucine and valine degradation are generated in *V. parahaemolyticus* VBNC cells. Thus, no increased synthesis of branched-chain fatty acids was detected. In the early stationary phase the degradation of isoleucine and valine was induced, and branched-chain fatty acids can be generated accordingly (O'LEARY, 1989).

FadR has a dual role in fatty acid metabolism and acts as a repressor in long-chain fatty acid transport, activation, and β -oxidation (NAVARRO LLORENS *et al.*, 2010; NYSTROM, 2004). The *fadR* operon is crucial for bacterial survival under starvation. The *fadR* operon activates the long-chain fatty acids catabolism but also involves in providing carbon and energy during the digestion of membrane constituents (FAREWELL *et al.*, 1996). The *fadE* encodes acyl-CoA dehydrogenase of the long-chain fatty acid β -oxidation pathway (CLARK and CRONAN, 1996). The β -oxidation pathway catabolises acyl-CoA, and generates acetyl-CoA as a source of carbon and energy (NYSTROM, 2004). In our study, VP2071 (*fadR*) showed no alteration in expression in the VBNC state and the early stationary phase. The genes (VP2289, VPA0616, VPA0622, VPA0623, VPA0624, VPA1119, VPA1120, VPA1121, VPA1127, VPA1152) encoding β -oxidation enzymes were significantly induced more than 5 times, while VP0029 and VP0030 showed no expression change in the early stationary phase compared with the exponential phase. However, the expression of β -oxidation enzymes encoding genes was diverse in the VBNC state compared with exponential and/or early stationary phases. Thus, data gathered in our study suggested that the β -oxidation pathway mediates acetyl-CoA

generation in the early stationary phase of *V. parahaemolyticus* in order to maintain bacterial survival. The β -oxidation pathway was not inhibited in *V. parahaemolyticus* VBNC cells, however, not all the genes showed up-regulated. Thus the fatty acid metabolism of *V. parahaemolyticus* VBNC cells might be reduced. Since fatty acids play an important role in cells at low temperatures, maintaining the membrane fluidity and the survival, the change of fatty acid metabolism allows maintenance of viability in the VBNC state (DAY and OLIVER, 2004).

When microorganisms are in unfavorable conditions, a stress response can be developed to adapt and survive in this environment. A number of specific stress response pathways, e.g. the general stress response and the heat-shock response, have been described in numerous bacteria species (AERTSEN and MICHIELS, 2005; POOLE, 2012). However, the stress response might be insufficient to protect bacterial cells against the stress. Hence, genetic diversity can be evolved by a number of mechanisms to enhance bacterial survival under stress (AERTSEN and MICHIELS, 2005). There are two major strategies that could manage the creation of genetic diversity. First, genetic diversity is generated during DNA replication and repair because of the small local changes in the genomic nucleotide sequence. Second, genetic diversity is mediated by recombinases and transposable elements because of the intra-genomic reshuffling of DNA fragments (ARBER, 2000). Many mechanisms, inducing genetic diversity, are important; however, two major mechanisms of concern methyl-directed mismatch repair (MMR) and SOS response in *V. parahaemolyticus* cells were found.

The MMR system is one of DNA repair pathways present in almost all life forms from bacteria to men (SCHOFIELD and HSIEH, 2003). MMR targets base-base mismatches and removes miss-incorporated bases in newly replicated DNA. Moreover, MMR plays an important role in guarding the genome. Inactivation of MMR function has been demonstrated to be associated with high frequencies of spontaneous mutation and recombination in diverse pathogenic bacteria (DENAMUR and MATIC, 2006; KIVISAAR, 2003; MATIC *et al.*, 1995). MutS, MutH and MutL are essential in detecting the mismatch and directing repair mechanism. MutS first recognizes the mismatches in double-stranded DNA followed by binding to it. Then MutL controls the excision of mismatches after cleavage by the MutH endonuclease. The strand which is cleaved and excised depends on the methylation state of the DNA. This system presumes that the unmethylated strand of a nascent and hemimethylated duplex DNA is the new strand, therefore the strand contains the mutation (FINKEL, 2006; SCHOFIELD and HSIEH, 2003). In our study, the expression of VP0518 (*mutH*), VP2552 (*mutS*) and VP2819 (*mutL*) was not changed in the early stationary phase compared with the exponential phase, indicating MMR expression was stable in the early stationary phase. Moreover, VP2552 showed no difference, while VP0518 and VP2819 were significantly up-regulated more than 6 times in *V. parahaemolyticus* VBNC cells, suggesting the MMR was induced (adjusted P -value $< 7 \times 10^{-7}$).

SOS response, addressing damaged DNA in several bacteria, is activated when DNA lesions are encountered during DNA replication (DA ROCHA *et al.*, 2008; SANCHEZ-ALBEROLA *et al.*, 2012). The SOS response is induced after cellular DNA damage, but it can also be induced whenever the concentration of active LexA is reduced. Thus, the global repressor LexA controls the induction of SOS genes. Direct regulation of SOS response is performed via *recA-lexA* (DA ROCHA *et al.*, 2008; SANCHEZ-ALBEROLA *et al.*, 2012). LexA recognizes a palindromic binding motif (SOS box), thereby inhibits the expression of enzymes correlated with DNA cleavage, recombination, and duplication (LITTLE and MOUNT, 1982; WERTMAN and MOUNT, 1985). The inactivation of LexA is a proteolytic reaction that is promoted by RecA protein. RecA induces the autocatalytic cleavage of the LexA dimer to prevent binding of LexA to SOS box, and hence activating the expression of SOS genes (LITTLE, 1991). In addition, the *imuA-imuB-dnaE2* mutagenesis cassette is also considered relating to SOS

response. The activity of this cassette is to induce mutagenesis and tolerate damage (ABELLA *et al.*, 2004; CAMPOY *et al.*, 2005; ERILL *et al.*, 2006; MAZON *et al.*, 2006; SANCHEZ-ALBEROLA *et al.*, 2012; WARNER *et al.*, 2010). As DNA arrangement and DNA damage happens in the VBNC state (FAKRUDDIN *et al.*, 2013; OLIVER, 2000a), an induction of the *imuA-imuB-dnaE2* mutagenesis cassette can be expected. In our study, the expression of VP2034 (*imuA*), VP2035 (*imuB*), VP2036 (*dnaE2*), VP2550 (*recA*) and VP2945 (*lexA*) was stably in the early stationary phase compared with the exponential phase, suggesting the SOS response was inactivated. However, VP2034, VP2035, VP2036 as well as VP2945 were significantly induced more than 5 times in the VBNC state along with genes encoding repair proteins (VP0518, VP0648, VP2570 and VP2819), while the VBNC state gene VP2550 was not considerably altered in expression. These findings indicate that SOS response might be activated via another pathway but *recA-lexA*. Additionally mutagenesis might be induced in the VBNC state. To sum up, genetic diversity has been created in the VBNC state via MMR and SOS response but not in the early stationary phase.

Biofilm is referred to as a polymer containing a community of microorganisms, which can be single microbial species or numerous microbial species, attaching to a range of biotic and abiotic surfaces (O'TOOLE *et al.*, 2000a). Biofilm formation depends on various environmental conditions, such as temperature, nutrient, pH, oxygen, iron and osmolality (O'TOOLE *et al.*, 2000b; PALMER and WHITE, 1997; PRATT and KOLTER, 1998; PRINGLE and FLETCHER, 1986; STOODLEY *et al.*, 1998; WANG *et al.*, 1996; WATNICK *et al.*, 1999; WATNICK and KOLTER, 1999; WIMPENNY and COLASANTI, 1997). Biofilms increase the resistance of bacteria to stresses e.g. UV light (O'TOOLE *et al.*, 2000a), altering the capability of biodegradation (KARAMANEV *et al.*, 1998), and increase the production of secondary metabolites (SARRA *et al.*, 1999).

In addition, many studies have reported biofilm raise the induction of the VBNC state (CERCA *et al.*, 2011; PASQUAROLI *et al.*, 2014; PASQUAROLI *et al.*, 2013; TREVORS, 2011). *C. jejuni* biofilm cells were found to enter the VBNC state faster than planktonic cells (MAGAJNA and SCHRAFT, 2015). AphA, a small PadR-family DNA-binding regulator, has been reported to be necessary for biofilm formation, virulence as well as swimming and swarming motility in *V. parahaemolyticus* (WANG *et al.*, 2013b). The tryptophanase encoded by *tnaA* degrades tryptophan into pyruvate, ammonia and indole in a reversible reaction (HU *et al.*, 2010; LEE *et al.*, 2012). Indole was found to be synthesized only when bacteria encode *tnaA* and to diminish the biofilm formation of *E. coli* (BANSAL *et al.*, 2007; HU *et al.*, 2010; LEE *et al.*, 2007; LEE *et al.*, 2012; LEE and LEE, 2010). Thus *tnaA* has been described to be of importance in biofilm formation. Likewise, a gene set encoding the mannose-sensitive haemagglutinin (MSHA) pilus is one of two known sets of type IV pilus genes. Because the MSHA pili contribute to the bacterial attachment to the surface, they seem to be important in biofilm formation of *V. parahaemolyticus* (SHIME-HATTORI *et al.*, 2006). The MSHA pilus genes, in combination with the gene cluster *pil*, affect biofilm formation in *Vibrio* spp. (MEIBOM *et al.*, 2004; PARANJPYE and STROM, 2005). In our study a significant up-regulation of VP2762 (*aphA*) and biofilm formation-related genes (VP2693 (*mshP*), VP2694 (*mshO*), VP2695 (*mshD*), VP2696 (*mshC*), VP2697 (*mshA*), VP2699 (*mshF*), VP2700 (*mshG*), VP2701 (*mshE*), VP2747 (*pilP*), VP2748 (*pilO*), VP2749 (*pilN*) and VP2750 (*pilM*)) was shown, indicating that the formation of biofilm was maintained under the VBNC state. VPA0192 (*tnaA*), however, was down-regulated in *V. parahaemolyticus* VBNC cells, indicating that no indole caused biofilm destruction happened. In contrast in the early stationary phase, VP2762 (*aphA*) and the biofilm formation-related genes mentioned above showed no expression changes, but VPA0192 (*tnaA*) was significantly induced approx. 6 times compared with exponential phase, suggesting that biofilm has not been formed in this phase.

Expression of various virulence factors has been reported in the VBNC state of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*, respectively (COLWELL *et al.*, 1996; FISCHER-LE SAUX *et al.*, 2002; VORA *et al.*, 2005). The findings of our study confirmed this. The expression of important *Vibrio* spp. virulence-associated genes: VP0819 (*toxS*), VP0820 (*toxR*), VPA1378 (*tdh1*) and VPA1314 (*tdh2*), was not reduced in the VBNC state. Except VP1401 and VPA1321 showed a reduction of expression in the VBNC state, other genes belonging to the T3SS family and T6SS family were not down-regulated. Since the global regulator (*rpoN*), which controls flagella synthesis, was significantly down-regulated by approx. 4 and 12 times, respectively, in the VBNC state compared with the exponential and the early stationary phases (adjusted *P*-value = 1×10^{-4} and 4×10^{-8}). The repression of *rpoN* results in a reduced synthesis of flagella. To sum up, *V. parahaemolyticus* VBNC cells maintain virulence since the virulence related genes are expressed stably. Although VBNC cells retain the capacity to cause disease, it remains unclear whether VBNC cells are sufficient to cause disease or if they must first resuscitate. Up to date the common theory is that pathogenic bacteria can infect humans after they resuscitate from the VBNC state under the suitable conditions (DU *et al.*, 2007; OLIVER, 2010; SUN *et al.*, 2008). Sun *et al.* (2008), for example, found that VBNC *V. harveyi* cells did not cause death but resuscitated cells were lethal when inoculated into zebra fish. Until now, resuscitation has been reported only in 26 species of human VBNC state pathogens, but it does not mean that other human pathogens could not resuscitate from VBNC state because of lacking suitable conditions of resuscitation. Only little is known of the mechanism of resuscitation and further research is needed.

In conclusion, this study has investigated the gene expression patterns of *V. parahaemolyticus* in exponential, early stationary phases as well as the VBNC state and showed that many genes elevate expression in the early stationary phase and the VBNC state. These distinct up-regulated expression patterns may have important roles for bacteria survival in the stationary phase and the VBNC state. The results presented in this thesis add new data towards an understanding of the mechanisms involved in the VBNC state.

Chapter 5 Summary

Vibrio spp. are isolated continuously from the estuarine, marine and sediment environments and seafood. The consumption of and/or contact to contaminated seawater or food can lead to infections in humans. *V. parahaemolyticus* can cause human gastroenteritis via consumption of contaminated raw or not thoroughly heated seafood.

In its natural habitat *V. parahaemolyticus* cell is forced to remain in the stationary phase or the viable but non-culturable (VBNC) state for survival.

The stationary phase is the common phase for bacterial survival in the environment. The metabolism of bacterial cells in the stationary phase is active but altered. Moreover, the resistance of stationary phase cells is greater to variety of stresses.

The viable but non-culturable (VBNC) state is referred to as enabling survival of especially non-sporulating bacteria, including *Vibrio* spp., in unfavourable or even hostile environments. The VBNC state can be induced by numerous factors, such as low temperature, depleted nutrient and adverse pH. Although bacterial VBNC cells are non-culturable on any routine laboratory medium, they maintain the ability of infection as the virulence genes are still expressed.

In this thesis, whole transcriptomic profiling of *V. parahaemolyticus* in the three phases (exponential and early stationary phase, the VBNC state) was performed. Studies on the global gene expression of *V. parahaemolyticus* demonstrated a broad adaptation of expression induced via the early stationary phase and the VBNC state. Among the 4820 investigated genes, 172 genes were induced while 61 genes were repressed totally. The altered processes were shown to be important for bacterial survival in the stationary phase against many stresses.

In the VBNC state compared with exponential and early stationary phase, up-regulated gene expression was also dominant. Totally 509 induced genes and 309 repressed genes among 4820 investigated genes were altered by more than fourfold. All these processes were shown to be of importance for the VBNC state maintenance. Massive regulations have been found, indicating that VBNC cells are active.

Until now it remains unclear what the incidence of food associated outbreaks caused by the food pathogenic VBNC state bacteria is. As the common detection methods depend on the CFU count and therefore growing cells, VBNC state bacteria avoid detection. Our research adds new data which might support the development of new methods for detection of not only *V. parahaemolyticus* VBNC cells but also other food pathogenic VBNC state bacterial cells.

Chapter 6 Zusammenfassung

Genexpressionsprofile von *Vibrio parahaemolyticus*

Vibrionen können kontinuierlich aus dem Meeres, dem Ästuar, dem Sediment und aus Meeresfrüchten isoliert werden. Der Kontakt mit Vibrionen enthaltendem Meerwasser und/oder der Verzehr von Lebensmitteln, die Vibrionen enthalten, sind die häufigsten Auslöser einer Vibriose beim Menschen. Die Spezies *Vibrio (V.) parahaemolyticus* ist humanpathogen und kann durch den Verzehr von kontaminierten, rohen oder nicht ausreichend erhitzten Meeresfrüchten zu Gastroenteritis führen.

Um das Überleben, in seinem natürlichen Lebensraum auch bei schlechten Umgebungsbedingungen zu sichern, ist *V. parahaemolyticus* in der Lage, in der stationären Phase zu verharren oder in einen Zustand überzugehen, indem das Bakterium lebensfähig, aber nicht kultivierbar ist (VBNC).

Die stationäre Phase gewährleistet ein Überleben von Bakterien in der Umwelt. Der Metabolismus von bakteriellen Zellen ist in dieser Phase aktiv aber im Vergleich zu anderen Phasen verändert. Die Zellen in der stationären Phase sind um einiges widerstandsfähiger und somit in der Lage verschiedenen Stressfaktoren besser Stand zu halten.

Vibrio spp. verfügen über die Möglichkeit, unter ungünstigen oder gar feindlichen Umgebungsbedingungen in diese Art von lebenserhaltendem Zustand überzugehen. Das VBNC Stadium kann durch unterschiedlichste Faktoren, wie niedrige Temperatur, absinkender Nährstoffgehalt, nicht optimaler pH-Wert, etc. induziert werden. Obwohl bakterielle VBNC Zellen auf keinem Routinemedium kultivierbar sind, besteht die Möglichkeit einer Humaninfektion, da Virulenzgene noch immer exprimiert werden.

In dieser Arbeit wurde das globale Transkriptom von *V. parahaemolyticus* in exponentieller und früher stationärer Phase, sowie im VBNC Stadium untersucht. Diese Studien, zur globalen Genexpression von *V. parahaemolyticus* zeigten, dass massive Anpassungen der Expression um mehr als das Vierfache während der frühen stationären Phase und dem VBNC Stadium stattfinden. Von den 4820 untersuchten Genen wurden 172 Gene induziert, während 61 Gene in ihrer Expression gehemmt wurden. Die hochregulierten Gene konnten hauptsächlich der Adaption des Bakteriums an die stationäre Phase zugeordnet werden. Die in der stationären Phase detektierten Veränderungen der Genexpression waren für das Bakterium essentiell, um ihr Überleben in der Umwelt zu sichern.

Beim Vergleich der Genexpression im VBNC Stadium mit der exponentiellen und frühen stationären Phase wurden 509 induzierte Gene und 309 unterdrückte Gene detektiert. Die induzierten Gene konnten mit dem Übergang in den VBNC Zustand assoziiert werden. Die Expression übergeordneter Regulatoren wurde induziert oder zumindestens stabil exprimiert. Alle diese Veränderungen spielen für die Aufrechterhaltung des VBNC Stadiums eine große Rolle. Aufgrund der drastischen Veränderungen die innerhalb der bakteriellen Regulatoren stattfanden, konnte nachgewiesen werden, dass VBNC Zellen aktiv sind.

Die Inzidenz der Lebensmittel-assoziierten Ausbrüche, die durch Bakterien im VBNC Stadium verursacht werden, bleibt unklar. Da herkömmliche Methoden auf dem Wachstum von Zellen beruhen, werden Bakterien im VBNC Stadium nicht detektiert. Unsere Arbeiten stellen neue Daten zur Verfügung, die die Entwicklung neuer Methoden nicht nur zur Detektion von *V. parahaemolyticus* im VBNC Stadium, sondern auch anderen pathogenen Lebensmittel-assoziierten Bakterien unterstützen könnten.

Chapter 7 References

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Chapter 8 Supplementary Materials

Fig. 8-1 Correlation of microarray and RT-qRT expression of selected genes in the VBNC state compared with exponential phase (log₂ transformed values) 102

Fig. 8-2 Correlation of microarray and RT-qRT expression of selected genes in the VBNC state compared with early stationary phase (log₂ transformed values) 103

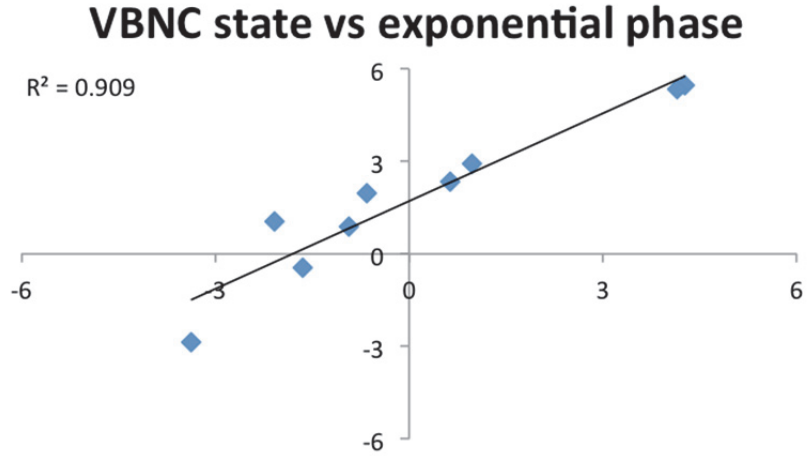


Fig. 8-1 Correlation of microarray and RT-qRT expression of selected genes in the VBNC state compared with exponential phase (log2 transformed values)

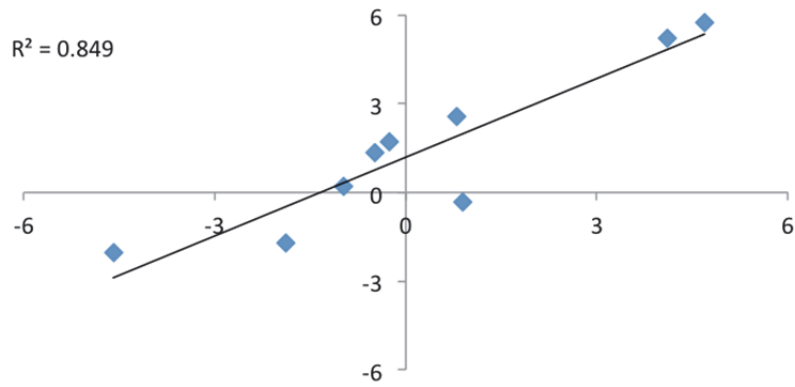
VBNC state vs early stationary phase

Fig. 8-2 Correlation of microarray and RT-qRT expression of selected genes in the VBNC state compared with early stationary phase (log₂ transformed values)

Chapter 9 List of Publications

Publications

Meng, L., Alter, T., Aho, T., Huehn, S. 2015. Gene expression profiles of *Vibrio parahaemolyticus* in viable but non-culturable (VBNC) state. FEMS Microb Ecol, 2015, 91(5): pii: fiv035.

Meng, L., Alter, T., Aho, T., Huehn, S. 2015. Gene expression profiles of *Vibrio parahaemolyticus* in early stationary phase. Lett Appl Microbiol, 2015, 61(3):231-237.

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Poster

Meng, L.; Alter, T.; Huehn, S. 2013. Induktion des VNBC (lebend aber nicht kultivierbaren)-Stadiums von *Vibrio parahaemolyticus*. 54. Arbeitstagung des Arbeitsgebiets Lebensmittelhygiene der Deutschen Veterinärmedizinischen Gesellschaft Garmisch-Partenkirchen – 24.09. - 27.09.2013.

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Chapter 10 Acknowledgements

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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.

Meng Lu

Berlin, 18.06.2015