10]; routine bacteriological examinations; and treatments at low temperature. These should be performed in slaughterhouses, transportation facilities and retail sites more frequently than are currently done by meat inspectors. Broad epidemiological surveillance is necessary for risk analysis by monitoring isolation of *Salmonella* spp. and antibiotic resistance at different stages of the pork production chain in cooperation with Vietnamese and Japanese experts.

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Identification and analysis of the osmotolerance associated genes in *Listeria monocytogenes*

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Listeria monocytogenes, the causative agent of listeriosis, has strong osmotolerance and is able to grow in severe circumstances. Many studies of the mechanisms of listerial osmotolerance have been performed. However, there is much which remains unknown. In previous studies we constructed two kinds of mutant in *L. monocytogenes* EGD strain to analyse the mechanisms of osmotolerance in *L. monocytogenes* by molecular genetic methods. In this paper, we summarized the genetical studies of osmotolerance in this bacterium by many researchers and ourselves. First, a transposon-insertional mutant strain was constructed that showed reduced growth in high osmotic agar compared with the parental strain. The results of cloning and sequencing analysis showed that the *rel* gene, which encodes guanosine tetra- and pentaphosphate synthesis and hydrolysis protein, is involved in osmotolerance in *L. monocytogenes*. Next, the expression levels of five sigma factor coding genes in *L. monocytogenes* were examined using real-time polymerase chain reaction (PCR) and it was found that the *rpoN* gene (the alternative sigma factor RpoN (sigma54)-encoding gene) was activated under high osmotic conditions. A deletion mutant of *rpoN* was constructed and its response to osmotic stress was analysed. In minimal medium with NaCl and carnitine, an osmoprotectant, the mutant showed deficient growth to that of the parental strain when the starting optical density was high, though the expression level of carnitine transporter operon, *opuC*, and the rate of carnitine uptake in the mutant was similar to that of EGD. These results suggest that the *rpoN* mutant may need larger amounts of carnitine which might be needed for its growth under high osmolarity. Through the analysis of these mutants, new insights have been obtained into osmotolerance in *L. monocytogenes*.

Keywords: *Listeria monocytogenes*; osmotolerance

Introduction

Listeria monocytogenes is a Gram-positive, spore non-forming bacteria and ubiquitous in the environment such as river water and intestines of wildlife animals or livestock (Iida et al. 1991; Inoue et al. 1991, 1992; Yoshida et al. 2000; Hayashidani et al. 2002). It is well known for its characteristics, ability of growth even at 4°C (Walker et al. 1990) and strong osmotolerance, which make listerial cells able to survive in very high osmolarity (Seeliger and Jones 1986). Because of these characteristics, prevention of food contamination in plants and inhibition of listerial growth in food stored in refrigerators are very difficult. Many kinds of food were reported as contaminated by this organism in Japan (Okutani et al. 2004b), similar to in Europe (Rudol and Scherer 2001; Beloeil et al. 2003) and the USA (Gombas et al. 2003; Lappi et al. 2004; Thimothe et al. 2004). In these countries, epidemics of listeriosis have occurred every two or three years (Jacquet et al. 1995; De Valk et al. 2001; Graves et al. 2005; Olsen et al. 2005). In Japan, most of cases of listeriosis were not part of an epidemic, and the food responsible for the infection could not be found in each case. A recent study showed that the cases of listeriosis are estimated to about 83 cases per year (Okutani et al. 2004a), and the occurrence rate was similar to the countries in Europe and USA. The first recognized epidemic case was reported in 2002 (Makino et al. 2005), caused by fresh cheese made in Japan. *L. monocytogenes* is sterilized by heat at 72°C for 1 min; however, human listeriosis is caused mainly by so-called ready-to-eat foods like fresh cheese, salad, and smoked salmon, and these kinds of food cannot be heated before they are consumed. The other ways to prevent the growth of bacteria that causes food-borne disease are refrigeration and the addition of chemical compounds inhibiting bacterial growth such as salt. An increase of extracellular osmorality prevents bacterial growth by injury to the membrane, reduction of water activity, and so on (Wood 1999). However, *L. monocytogenes* can grow even in media supplemented with 12% NaCl and can survive under 20% NaCl (Seeliger and Jones 1986). Based on this background knowledge, we studied the mechanisms of strong osmotolerance in

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L. monocytogenes by molecular genetic methods. Here we report our previous studies summarizing the genetic analysis of osmotolerance by many researchers who have worked on this bacterium.

Listerial osmotolerance-associated genes

The mechanisms of its strong tolerance to osmotic stresses are partially clarified at present. Osmotolerance-associated genes reported are shown in Table 1. The mutants of these genes showed reduced growth under high osmotic condition. Like many other species of bacteria (Csonka et al. 1994; Lin and Hansen 1995), *L. monocytogenes* accumulates so-called osmoprotectants or compatible solutes including glycine-betaine (betaine), carnitine and proline (Ko and Smith 1999; Sleator et al. 1999; Fraser et al. 2000; Sleator, Gahan et al. 2001, Sleator, Wouters et al. 2001) under high osmotic conditions. Betaine has the strongest effect on listerial growth under high osmotic pressure amongst these three chemicals (Beumer et al. 1994). Furthermore, the expression of genes encoding these transporters are transcriptionally regulated by general stress responsible sigma factor, σ^B (Sleator et al. 2003a). In the analysis of BetL, the secondary transporter of betaine in *L. monocytogenes*, Sleator et al. constructed a mutant that had a σ^B-independent, osmotically non-inducible promoter instead of the original, σ^B-dependent, osmotically inducible promoter at upstream of *betL* gene; however, the mutant showed the osmotic induction of betaine uptake (Sleator et al. 2003b). From these, it is supposed that some regulatory factors of osmoprotectant coding genes translation or osmoprotectant uptake are existent in *L. monocytogenes*.

Most listerial osmotolerance-associated genes have other functions in stress tolerance such as low temperature, acid tolerance and virulence in mice (Table 1). In our previous study, a transposon (Tn)-insertional mutant, which showed an osmosensitive phenotype, was obtained (Okada et al. 2002). From the Tn-inserted region in listerial choromosome, a 2214-bp open reading frame was identified as *rel*, which encodes guanosine tetra- and pentaphosphate (collectively called as (p)ppGpp) synthetase. It was found that the mutant was able to use the extracellular osmoprotectant like its parental strain, and the basal level of (p)ppGpp before exposure to osmotic stress was important for osmotolerance in *L. monocytogenes*. ppGpp is known to be a positive regulator of a the σ^B-homologue, RpoS, in *Escherichia coli* (Gentry et al. 1993), and it corresponds to regulation of the expression of many genes under stress conditions such as nutrition limitation and stationary phase growth (Trexler et al. 2006). We are currently performing a continuous study on the relationship between *rel* and σ^B on osmotolerance in *L. monocytogenes*.

Sigma factors in *L. monocytogenes*

In general, bacterial survival under stress conditions requires rapid alterations in gene expression, controlled by the association of different alternative sigma factors with core RNA polymerase (Jishage et al. 1996). Previous studies have reported the existence of four types of sigma factors in *L. monocytogenes*, including RpoD, σ^B, σ^H, and RpoN. In addition to these sigma factors, the available evidence suggests that EGD contains at least one extra-cytoplasmic functional (ECF)-type sigma factor as evidenced by an analysis using the Database of PEDANT of the Munich Information Center of Protein Sequences (Okada et al. 2006) (Table 2). Zhang et al. reported that the deletion mutant of ECF sigma factor-coding gene, lmo0423, showed loss of thermal adaptation in 10403S strain (Zhang et al. 2005). In *L. monocytogenes*, RpoD has been recognized as a principal sigma factor (Metzger et al. 1994), and σ^B has been recognized as a general stress-responsive sigma factor that contributes to survival under several adverse conditions, such as high-osmolarity conditions (Becker et al. 1998), low temperature (Becker et al. 2000) and acidic conditions (Wiedman et al. 1998). In *L. monocytogenes*, σ^H encoded by *sigH* is induced in *L. monocytogenes* by low pH (Phan-Thanh and Mahouin 1999) and the *sigH* mutant in *L. monocytogenes* was impaired in growth under alkaline conditions and affected in growth in minimal medium (Rea et al. 2004). In *Bacillus subtilis*, σ^H is known as a stationary phase sigma factor (Haldenwang 1995), and σ^L, which is highly homologous to RpoN encoded by *rpoN* in *Listeria* and σ54 in Gram-negative bacteria, is involved in the regulation of amino acid metabolisms (Debarbouille et al. 1991) and the acetoin carbolic pathway (Ali et al. 2001). A *B. subtilis sigL* mutant showed reduced growth rate when arginine, ornithine, isoleucine or valine was supplied as a nitrogen source (Debarbouille et al. 1991). RpoN and sigmaL in Gram-positive bacteria and σ54 in Gram-negative bacteria are distinct from sigma70-type sigma factors. The former sigma factors bind to $-24/-12$ promoters, while sigma 70-type sigma factors bind $-35/-10$ promoters (Morett and Buck 1989; Merrick 1993; Burrows et al. 2003). Furthermore, sigma54-dependent RNA polymerase needs an activator, bacterial enhancer binding proteins (EBPs) to initiate transcription (Buck et al. 2000). Studholme and Dixon (2003) reported that *L. monocytogenes* has three kinds of EBP, as deduced from its complete genome sequence, and two of these proteins were suggested to be controllers of phosphotransferase systems (PTS). Robichon et al. (1997) have reported that RpoN in *L. monocytogenes* is involved in bacteriocin resistance. Subsequent studies by the same group showed that a σ54-associated activator, ManR, and a σ54-dependent PTS permease of the mannose familv.

Table 1. Osmotolerance associated genes in *Listeria monocytogenes*.

Gene/operon	Size (bp)	Characteristics of product	Other functions of resistance	References
Osmoprotectant transporter				
betL (lmo2092)	1524	Betaine transporter	Low temperature	Sleator et al. (2003b); Wemekamp-Kamphuis et al. (2004a)
gbu (lmo1014–1016)	1194 + 849 + 903	Betaine transporter	Low temperature	Ko et al. (1999); Wemekamp-Kamphuis et al. (2004a)
opuC (lmo1428–1425)	1194 + 657 927 + 672	Carnitine transporter	Low temperature	Fraser et al. (2000) Wemekamp-Kamphuis et al. (2004a)
Osmoprotectant synthetase				
proBA (lmo1260–1259)	831 + 1248	Proline synthetase	Virulence in mouse	Sleator et al. (2001)
General stress-responsive factor				
sigB (lmo0895)	780	Stress responsive-sigma factor	Acid, high hydrostatic pressure, freeze	Becker et al. (1998, 2000); Wiedman et al. (1998); Wemekamp-Kamphuis et al. (2004b)
clpC (lmo0232)	2463	Endopeptidase Clp ATP binding chain C	Iron limitation, high temperature	Rouquette et al. (1996)
ctsR (lmo0229)	459	Transcriptional repressor	High temperature	Nair et al. (2000)
hfq (lmo1295)	234	RNA-binding protein	Ethanol, stationary phase	Christiansen et al. (2004)
dtpT (lmo0555)	1479	Di- and tripeptide transporter	Virulence in mouse	Wouters et al. (2005)
htrA (*degP*) (lmo0292)	1503	Serine protease	High temperature, heat shock, H_2O_2	Wonderling et al. (2004)
kdpE (lmo2678)	676	Two-component response regulator	–	Brondsted et al. (2003)
rel (lmo1523)	2217	(p)ppGpp synthetase	Stringent response	Okada et al. (2002)
rpoN (lmo2461)	1344	RNA polymerase sigma54	Bacteriocin	Okada et al. (2006)

Table 2. Sigma factors in *Listeria monocytogenes*.

σ factor	Coding gene (bp)	Known function	Related σ factor in *B. subtilis* (function) Putative promoter sequences	References
RpoD (σ43)	*rpoD* (1125)	General	σA (general)	Metzger et al. (1994)
TTGACA, 17, TATAAT SigB (σ37)	*sigB* (780)	Stress responses	σB (stress response)	Becker et al. (1998, 2000); Wiedmann et al. (1998)
RGGNTTRA, 14, GGGTAT SigH (σ30)	*sigH* (606)	Acid tolerance	σH (stationary phase)	Rea et al. (2004)
RVAGGANNT, 14, MGAAT RpoN (σ54)	*rpoN* (1344)	Bacteriocin resistance	σL (nitrogen utility)	Robichon et al. (1997)
TGGCA, 5, CTTGCANNN ECF type σ factor (σ70 family) TGAACG, N	lmo0423 (549)	Heat shock	ECF-type sigma factor (stress response)	Zhang et al. (2005)

Notes: Nucleotide abbreviation: M = A or C; R = A or G; V = A, C or G; N = A, G, C or T.
Reference for *B. subtilis*: Haldenwang (1995).

EII^{Man}_{t}, are involved in bacteriocin sensitivity (Dalet et al. 2001). Another PTS operon of the lactose family in *L. monocytogenes* strain LO28 was also shown to have a σ54-dependent promoter (Dalet et al. 2003). Arous et al. (2004) performed whole-genome microarray and proteome analysis of an *rpoN* mutant, and revealed that the expressions of 77 genes and nine proteins were modulated by RpoN. Most of them were related to carbohydrate metabolism. However, in a recent study, RpoN showed involvement in osmotolerance in *L. monocytogenes* (Okada et al. 2006). The deletion mutant of *rpoN* showed reduced growth in minimal medium with carnitine under high osmotic condition compared with the parental strain. However, RpoN did not directly regulate the expression of carnitine transporter operon, *opuC* and carnitine transporter rates. This mutant showed the similar growth with the parental strain when starting optical density of bacterial cells was low, so it was concluded that the *rpoN* mutant needs a higher amount of carnitine to grow under high osmotic condition than its parental strain. To identify the intact genes associated with growth under high osmotic condition and regulated by RpoN, proteome analysis is currently being performed.

Both osmitic stress-responsible genes examined in our previous study were also associated with the resistance to other kinds of stress, such as nutrient limitation or bacteriocin resistance in *L. monocytogenes*. This indicates that listerial osmotolerance is involved in the cross-protection to many kinds of environmental stresses and this help *L. monocytogenes* to survive in severe circumstances. Through the further analysis of listerial osmotolerance we hope to contribute to improved food hygiene.

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Antimicrobial resistance of *Campylobacter*: prevalence and trends in Japan

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Campylobacter is one of the most frequently diagnosed bacterial causes of human gastroenteritis in Japan and throughout the world. Resistance to quinolones in *Campylobacter jejuni* and *C. coli* isolated from humans has emerged in many countries during the past 15 years because fluoroquinolones are the drug of choice for the treatment of suspected bacterial gastroenteritis. Food contaminated with *Campylobacter* is the usual source of human infection; therefore, the presence of antimicrobial resistance strains in the food chain has raised concerns that the treatment of human infections will be compromised. The use of antimicrobial agents for food animals and in veterinary medicine is suspected to be correlated with an increase in quinolone-resistant strains of *Campylobacter* in food animals, especially in poultry products. In contrast to macrolide resistance in *C. jejuni* and *C. coli* isolated from humans showing a stable low rate, resistant *Campylobacter* spp. to quinolones have emerged in Japan. The paper summarizes food-borne *Campylobacter* infection in Japan, and the prevalence and trends of antimicrobial resistance of *Campylobacter* from the authors' data and other Japanese papers which reported the antimicrobial resistance of *Campylobacter*.

Keywords: microbiology; animal products; veterinary drug residues – antibiotic resistance; veterinary drug residues – fluoroquinolones; animal products; meat

Introduction

Campylobacter frequently causes enteric disease in humans. Most cases of *Campylobacter* infections are epidemiologically linked to the consumption of poultry products. *C. jejuni* is often present in retail poultry and is less frequently found in fresh beef, lamb, and pork. It is estimated that more than 50% of sporadic cases of *Campylobacter* enteritis are linked to eating or handling poultry (Annan-Prah and Janc 1988), and reducing the carriage of *C. jejuni* in poultry should have a major impact on decreasing human *Campylobacter* enteritis (Harris et al. 1986). An increase in the number of *Campylobacter* isolates resistant to quinolone in patients and animals has been reported (Engberg et al. 2006). Antimicrobial drugs are used in animals to control, prevent, and treat infection and, in food-producing animals, to enhance animal growth. Several epidemiological studies have discussed the relationship between the use of quinolone in animals and an increase in quinolone-resistant *Campylobacter*. We will summarize *Campylobacter* infections and the antimicrobial resistance of *Campylobacter jejuni/coli* in Japan.

Outline of *Campylobacter jejuni* and *C. coli* food-borne infections in Japan

In Japan there is the surveillance system for food-borne diseases in compliance with the Food Sanitation Law. This surveillance programme covers all the population of Japan and the listed food-borne pathogens including *Salmonella typhi*, *Salmonella paratyphi A*, other *Salmonella* spp., *Staphylococcus aureus*, *Clostridium perfringens*, *Clostridium botulinum*, *Vibrio parahaemolyticus*, *Vibrio cholerae*, enteropathogenic *Vibrio* spp., enterohaemorrhagic *Escherichia coli*, other enteropathogenic *E. coli*, *Shigella* spp., *Bacillus cereus*, *Yersinia enterocolitica*, *Campylobacter jejuni/coli*, and other pathogenic bacteria. When an outbreak of food-borne illness occurs, the local public health centre is responsible for conducting epidemiological studies and laboratory tests needed to determine the causes of infection. Those results are reported to the Ministry of Health, Labor and Welfare (MHLW) through the head office of the local government. Table 1 shows the results of the surveillance of food-borne diseases in 2005 in Japan (Inspection and Safety Division, MHLW 2006). *Salmonella* spp., *Vibrio parahaemolyticus* and *Campylobacter jejuni/coli* are the most important food-borne pathogens. Recently, the number of cases caused by *Clostridium perfringens* has been increasing, and we are more closely monitoring this increase. We have experienced several large outbreaks caused by *C. perfringens* in which the number of patients exceeded 500. *Campylobacter* species are the most frequently isolated pathogens

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Table 1. Japanese surveillance of food-borne diseases in 2005. Data are from the epidemiological data of food-borne disease in Japan 2005[4] (MHLW) (2006).

	Outbreaks	Cases	Deaths
Salmonella spp.	144	3700	1
Staphylococcus aureus	63	1948	–
Clostridium boturinum	–	–	–
Vibrio parahaemolyticus	113	2301	–
Shiga toxin-producing *E. coli*	24	105	–
Other enteropathogenic *E. coli*	25	1734	–
Clostridium perfringens	27	2643	–
Bacillus cereus	16	324	–
Yersinia enterocolitica	–	–	–
Campylobacter jejuni/coli	645	3439	–
Vibrio cholerae	–	–	–
Other *Vibrio* spp.	–	–	–
Shigella spp.	–	–	–
Others	8	484	–
Total	1065	16,678	1

from sporadic human enteritis in Japan. Figure 1 shows the isolation reports of *Campylobacter jejuni/coli* enteritis designated as a food poisoning in Japan. From 1999 to 2005, the number of both patients and incidents of enteritis caused by *Campylobacter* slightly increased (Figure 1). Up until 1999–2001, there were about 1800 *Campylobacter* food poisoning cases per year. However, this number exceeded 2000 in 2002, and increased to 3439 in 2005. Single-case incidents account for more than 70% of *Campylobacter* food poisoning incidents This is because some municipalities started to report single-case food poisoning incidents in 1997. Data are reported monthly in Japan (data not shown). Although outbreaks caused by other pathogens tend to increase in summer months, food-borne illness caused by *Campylobacter* occurs throughout the year.

When *Campylobacter* enteritis occurs, *Campylobacter* is easily isolated from patients, but it is more difficult to isolate this organism from foods. A Japanese paper reported that of 464 patients in which *Campylobacter* had been isolated, *Campylobacter* was also isolated from food in only 55 incidents. This report revealed that many cases of *Campylobacter* infections are epidemiologically linked to the consumption of poultry products or poultry-related food.

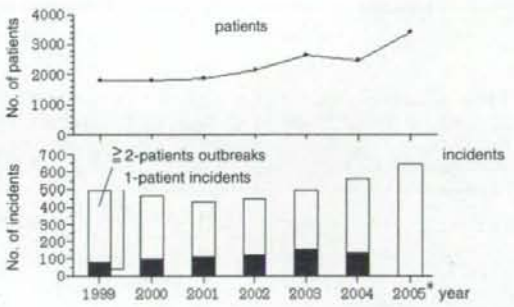

Figure 1. Food-borne cases of *Campylobacter jejuni/coli*: number of patients and incidences in Japan during 1999–2005. Data are from Infectious Agents Surveillance Report (IASR) (2006). *Data for 2005 are not finally fixed.

Antimicrobial resistance of *Campylobacter* isolated clinically

Campylobacter infections are recognized as one of the frequent causes of human sporadic diarrhoea. As noted above, poultry products are the most frequent infectious sources of human *Campylobacter* infection. In Japan, the number of nalidixic acid-resistant *Campylobacter* isolated from human sporadic diarrhoea has remarkably increased in number (Infectious Agents Surveillance Report (IASR) 1999, 2006). The *Campylobacter* Reference Center of Japan (CRCJ) consists of seven local public health institutes such as Akita, Tokyo, Aichi, Osaka, Hiroshima, Yamaguchi and Kumamoto, and the National Institute of Health Sciences located in Tokyo, which collect *C. jejuni/coli* isolates to perform serotyping and drug susceptibility tests. CRCJ conducts serotyping of clinically isolated *C. jejuni/coli* by the Lior system and studies the antimicrobial resistance of *Campylobacter*. A surveillance study of this reference centre was supported by grants from MHLW. Serotyping results of isolates from sporadic cases are listed in Table 2. Serotypes (Table 2) and antimicrobial susceptibilities (Table 3) of clinical isolates are provided. During 1998–2004, 4596 strains of *C. jejuni* isolated from sporadic diarrhoea cases were subjected to serotyping assay. A total of 2930 strains were typeable by a single

Table 2. Lior typing of *Campylobacter* isolates from sporadic cases between 1998 and 2004. Data are from reference Infectious Agents Surveillance Report (IASR) (2006).

Serovar	1998	1999	2000	2001	2002	2003	2004	Total	%
LIO 1	33	19	30	18	19	19	21	159	3.5
LIO 2	34	15	28	35	21	12	13	158	34.4
LIO 4	71	45	57	103	128	148	191	743	16.2
LIO 7	46	45	54	47	38	41	37	308	6.7
LIO 11	20	14	21	21	31	24	17	148	3.2
LIO 27	22	5	16	19	19	7	9	97	2.1
LIO 28	16	3	27	10	28	12	26	122	2.7
LIO 36	14	12	29	21	17	12	17	122	2.7
LIO 50	25	11	13	9	17	14	14	103	2.2
TCK 1	17	12	24	15	7	13	12	100	2.2
TCK 12	27	31	16	16	20	16	22	148	3.2
TCK 13	11	5	6	17	16	6	13	74	16.6
TCK 26	11	29	21	9	9	9	24	112	2.4
Others	83	60	77	64	79	92	81	536	11.7
Subtotal	430	306	419	404	449	425	497	2930	
(%)	62.8	64.6	65.7	61.7	60.1	63.7	68.1	63.8	
Multi	34	32	29	44	28	24	10	201	4.4
Unidentified	221	136	190	207	270	218	223	1465	31.9
Total	685	474	638	655	747	667	730	4596	100

Table 3. Antimicrobial resistance pattern of isolates from sporadic cases of human *Campylobacter jejuni* enteritis in 1997. *Susceptible to all tested antimicrobials. Resistance was tested with five drugs: norfloxacin (NFLX), ofloxacin (OFLX), ciprofloxacin (CPFX), nalidixic acid (NA), and erythromycin (EM). Data are adapted from Infectious Agents Surveillance Report (IASR) (1999).

	Resistant characteristics	Akita $n=53$	Tokyo $n=103$	Aichi $n=30$	Osaka $n=64$	Hiroshima $n=71$	Yamaguchi $n=44$	Kumamoto $n=57$	Total $n=422$	Relative (%)
1	NFLX						1		1	
	CPFX		1				2		3	
	EM	2	3			2			7	
2	NFLX, OFLX					2			2	
	NFLX, NA		3						3	
3	NFLX, OFLX, CPFX,				1	1	4		6	
	NFLX, OFLX, NA,		2	1			1		4	
4	NFLX, OFLX, CPFX, NA	16	25	8	22	21	13	8	113	(27%)
5	NFLX, OFLX, CPFX, NA, EM		1		2	2	1		6	
	Susceptible* (%)	35 (66)	64 (66)	21 (70)	39 (61)	45 (63)	20 (46)	49 (86)	277(66)	

serotype. The LIO4 type was the largest in number, 743 strains, 16%, followed by 308 strains, 6.7%, of the LIO7 type; 201 strains (4.4%) were typeable by more than two serotypes, and about 32% of isolates were not typeable by our serotype systems. During 1998–2004, 4183 strains of *Campylobacter*-derived sporadic diarrhoea cases were tested for resistance to six drugs including norfloxacin (NFLX), ofloxacin (OFLX), ciprofloxacin (CPFX), nalidixic acid (NA), tetracycline (TC), and erythromycin (EM). Several isolates showed some resistance to one or more of the antimicrobials (Table 3); however, of the 4183 strains tested, 2216 of these were susceptible to all tested drugs (53%) (data not shown). Yearly reports of the antimicrobial resistance of *Campylobacter* isolated from sporadic cases from 1998 to 2004 are shown in Figure 2.

During 1998–2004, 4183 strains of *Campylobacter*-derived sporadic diarrhoea cases underwent the drug susceptibility test. Resistance was tested by using disks (KB method) of six drugs including NFLX, OFLX, CPFX, NA, TC, and EM. Resistance was determined by the Kirby–Bauer method using BD Sensi-Disk (Japan Beckton Dickinson Co., Fukushima, Japan) with a diameter of growth inhibition zone as follows: NFLX, <13 mm; OFLX, <13 mm; CPFX, <16 mm; NA, <19 mm; TC, <15 mm; and EM, <14 mm. A total of 30–45% isolates were tetracycline resistant, and 25–40% isolates were nalidixic acid-resistant and/or fluoroquinolone-resistant. On the other hand, erythromycin resistance was very rare: 1–3%. The antimicrobial resistance of *Campylobacter jejuni* isolates from sporadic cases in 1997 is listed in Table 3.

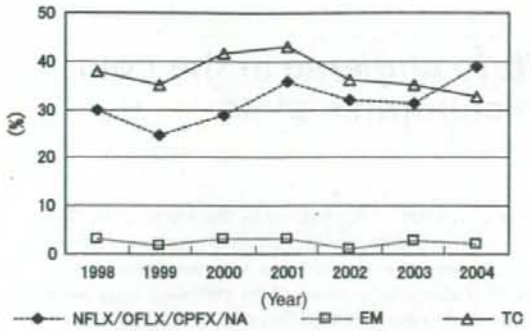

Figure 2. Antimicrobial resistance of isolates from sporadic cases of human *Campylobacter* enteritis. Resistance was tested with six drugs: norfloxacin (NFLX), ofloxacin (OFLX), ciprofloxacin (CPFX), nalidixic acid (NA), tetracycline (TC), and erythromycin (EM). Data are from Infectious Agents Surveillance Report (IASR) (2006).

Table 4. Relative antibiotic-resistant rates in *Campylobacter jejuni* isolates from clinical specimens in 1997. Resistance was checked with five drugs: norfloxacin (NFLX), ofloxacin (OFLX), ciprofloxacin (CPFX), nalidixic acid (NA), and erythromycin (EM). Data are adapted from Infectious Agents Surveillance Report (IASR) (1999).

	Human clinical isolates*
Tested	422
NFLX	129 (31)
OFLX	125 (30)
CPFX	122 (29)
NA	120 (28)
EM	7 (2)
Susceptible to all tested antimicrobilals	277 (66%)

These data have been reported by CRC. From these data, we can see different resistant patterns and the multi-drug resistance of each tested point.

The antibiotic-resistant rates of *Campylobacter jejuni* isolates from clinical specimens are listed in Table 4.

Conclusions

Japanese surveillance for food-borne diseases has demonstrated that *Campylobacter jejuni/coli* are the most important food-borne pathogens in Japan. Up until 1999–2001, there were about 1800 *Campylobacter* food poisoning cases per year. However, this number exceeded 2000 in 2002, and increased to 3439 in 2005. *Campylobacter* species are also the most frequently isolated pathogens from sporadic human enteritis. *Campylobacter* infections are epidemiologically linked to the consumption of poultry products or poultry-related foods. CRCJ serotyping and antimicrobial susceptibility testing of *Campylobacter* strains clinically isolated from sporadic diarrhoea cases during 1998–2004 showed that the LIO4 serotype was the most prevalent, followed by the LIO7 serotype. Antimicrobial susceptibilities of isolates were tested by using disks (KB method) containing six drugs: NFLX, OFLX, CPFX, NA, TC, and EM. A total of 30–45% isolates were tetracycline resistant, and 25–40% isolates were nalidixic acid resistant and/or quinolones resistant. On the other hand, erythromycin resistance was very rare: 1–3%. About 30% of human clinical isolates were multidrug-resistant strains. Most of multidrug-resistant *Campylobacter* were quinolones-resistant strains.

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Gene expression profile of *Vibrio cholerae* in the cold stress-induced viable but non-culturable state

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Summary

***Vibrio cholerae* is an aetiological agent of cholera that inhabits marine and estuarine environments. It can survive harsh environments by entering the viable but non-culturable (VBNC) state, but the related changes in gene expression have not been described. Here, we experimentally induced the VBNC state in *V. cholerae* O1, by incubation in artificial seawater at 4°C. Bacterial cells that were incubated for 70 days retained their membrane integrity and were pathogenic, colonizing the gut of iron-dextran-treated mice, even though they formed no colonies on tryptic soy agar (TSA) or TSA amended with pyruvate. We therefore used this stage of cells as the VBNC bacteria. We compared the global transcription pattern of the VBNC cells with that of stationary-phase cells grown in rich medium. A total of 100 genes were induced by more than fivefold in the VBNC state, and the modulated genes were mostly those responsible for cellular processes. Furthermore, real-time RT-PCR analysis verified the changes in the expression levels, showing that the VC0230 [iron(III) ABC transporter], VC1212 (*polB*), VC2132 (*fliG*) and VC2187 (*flaC*) mRNAs were increased in the non-culturable state. Thus, these genes may be suitable markers for the detection of VBNC *V. cholerae*. To our knowledge, this is the first report of a comprehensive transcriptome analysis of *V. cholerae* in the VBNC state. The significance of this gene expression profile compared with those of *in vivo* isolates and non-stressed bacteria (culturable *in vitro*) is its potential to provide information about the public health risk from dormant bacteria.**

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Introduction

Vibrio cholerae is an aetiologic bacterial agent of cholera, a severe diarrhoeal disease, especially endemic in the developing world. The bacteria inhabit marine and estuarine waters. *V. cholerae* grows in low-salinity environments when the water temperature is relatively high and organic nutrients are present in high enough concentrations to compensate, to some degree, for the lack of salt (Singleton *et al.*, 1982a,b; Huq *et al.*, 1984). Upon exposure to an unfavourable environment, the microorganism can survive by entering a dormant state, that is, the viable but non-culturable (VBNC) state (Roszak and Colwell, 1987). Bacteria in the VBNC state fail to grow on routine bacteriological media on which they would normally form colonies, but are alive and capable of renewed metabolic activity (Oliver, 2005). Like other bacterial species, *V. cholerae* can be made to enter the VBNC state experimentally (Wai *et al.*, 1996; Pruzzo *et al.*, 2003), and the ecology of the VBNC *V. cholerae* in water (Binsztein *et al.*, 2004) is relevant to public health, because of the likelihood that the bacteria will be resuscitated and start dividing upon access to an animal host (Oliver and Bockian, 1995; Asakura *et al.*, 2002; Baffone *et al.*, 2003; Coutard *et al.*, 2005). In fact, the pathogenicity of the dormant bacteria was initially demonstrated using ligated ileal loop assays (Grimes *et al.*, 1986), and its pathogenicity in humans was demonstrated in volunteer feeding experiments; therefore, VBNC *V. cholerae* can remain pathogenic, even after long-term residence in the environment (Islam *et al.*, 1989). Furthermore, the occurrence of the VBNC state in the bacteria may relate to the seasonal epidemics of cholera, because bacteria can adapt to protozoan predation (Matz and Kjelleberg, 2005). Despite these public health concerns, the molecular mechanisms of the VBNC state in bacteria have not been well characterized; the only evidence so far is regarding the stability of the mRNAs for some genes (Fischer-Le Saux *et al.*, 2002; Coutard *et al.*, 2005).

Recently, Gonzalez-Escalona and colleagues (2006) presented the interesting finding that the incubation temperature significantly affects the gene expression profile of *V. cholerae*. These authors compared the transcription of some target genes in the VBNC (induced at 4°C) and starvation (15°C) states, and showed that key enzymes for cellular metabolism, such as *tuf*, which is needed for protein synthesis, and *relA* or *rpoS*, which are stress-response genes, were detected at higher levels in bacteria that had entered the VBNC state.

Such findings strongly support the idea that the VBNC cells retain viability, and emphasize the importance of elucidating the survival mechanism(s) of the dormant bacteria. However, little is known about the genetic modulation induced in the VBNC-entering process of this organism in a foreign environment.

Given this background, we sought to understand changes in gene expression by bacteria during the VBNC state, with the expectation that we would glean important insights into microbial ecology and its transmission from an environmental source.

In this study, we experimentally induced the VBNC state in *V. cholerae* by growing it in artificial seawater (ASW) at 4°C, and its *in vivo* resuscitation was examined. Subsequently, we evaluated the transcriptional profile of *V. cholerae* VBNC cells compared with culturable cells using comparative microarray analysis, which annotated data for 3707 genes from the *V. cholerae* O1 strain N16961 chromosome. We found significant changes in mRNA expression in dormant *V. cholerae*. In addition, the mRNA expression levels for representative genes were monitored over time, and their changes with respect to alterations in bacterial culturability and membrane integrity are discussed.

Results and discussion

Generation of the VBNC state of V. cholerae *in ASW*

To study the process by which *V. cholerae* enters the VBNC state, we performed a comparative analysis of the viability kinetics of bacterial cells that were incubated in ASW using three different methods, essentially as described in Gonzalez-Escalona and colleagues (2006) with minor modifications: the first two involved determination of the number of cfu ml^{-1} by agar plating to establish the number of culturable cells that could grow on either (i) conventional culture media (tryptic soy agar, TSA) or (ii) TSA amended with sodium pyruvate (TSA-SP), which provides protection against reactive oxygen species by degrading non-enzymatic peroxide, and thereby promotes the recovery of starvation-stressed cells (Mizunoe *et al.*, 1999), (iii) the third used a LIVE/DEAD BacLight bacterial viability kit to determine the number of viable cells (i.e. bacteria with an intact membrane), independent of their growth potential.

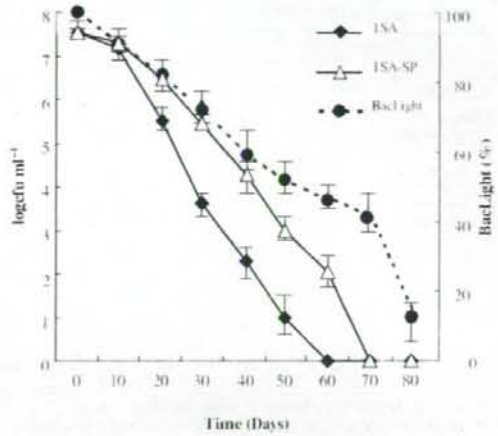

Fig. 1. Survival of *V. cholerae* in ASW. Culturable *V. cholerae* O1 cells in ASW incubated at 4°C for 60, 70 and 80 days, were counted by plating on TSA (solid diamonds) and TSA-SP (open triangles). In the simultaneous BacLight assay, green fluorescent cells were monitored by epifluorescence, and the percentages of green fluorescent cells among more than 1500 total cells from eight fields are shown (solid circles). The test was performed in triplicate and the data indicate means ± standard deviations.

When incubated in ASW at 4°C, *V. cholerae* strain P6973 lost its culturability on TSA, but on TSA-SP, 1.4×10^2 cells were recovered at 60 days post incubation (Fig. 1), suggesting that the injured bacteria had been recovered by the SP. The culturability on TSA-SP disappeared at 70 days post incubation (Fig. 1). In parallel, the membrane integrity of this strain decreased transiently. However, 43.8% of the cells retained their membrane integrity at 70 days post incubation (Fig. 1), suggesting that VBNC cells made up at least part of the non-culturable cell population. The membrane integrity had decreased significantly (10.8%), and no colonies formed on either media, at 80 days post incubation (Fig. 1), indicating that almost all of the bacteria had died.

In vivo *resuscitation of* V. cholerae *from the VBNC state in iron-dextran-treated mice*

Vibrio cholerae cells can be resuscitated from dormancy by heat shock (Wai *et al.*, 1996), but direct resuscitation *in vivo* has not been demonstrated. As *Salmonella* cells are directly resuscitated from the dormant state *in vivo* in morphine-treated mice (Asakura *et al.*, 2002), we predicted that *V. cholerae* might also be resuscitated *in vivo*.

To examine this issue, the pathogenicity of the ASW-incubated *V. cholerae* cells was examined in iron-dextran-treated mice. First, to estimate the LD_{50} (as a control) 30

Table 1. Virulence of the VBNC *V. cholerae* cells in iron-dextran-treated mice.

Total cell numbers ml^{-1}	Time (days)	Cfu		BacLight (%)[a]	Mice virulence		
					Number of mice		
		TSA	TSA-SP		Killed/tested	Colonized/tested	*V. cholerae* (cfu g^{-1} of caecum)[b]
Stationary							
2.1×10^6	0	2.1×10^6	NT	> 99.9	5/5	5/5	$> 10^8$
2.4×10^4	0	2.4×10^4	NT	> 99.9	5/5	5/5	3.3×10^7
4.5×10^3	0	4.5×10^3	NT	> 99.9	3/5	5/5	4.5×10^6
3.8×10^2	0	3.8×10^2	NT	> 99.9	1/5	5/5	7.9×10^5
2.8×10^1	0	2.8×10^1	NT	> 99.9	0/5	0/5	1.8×10^1
Saline	0	0	0	NT	0/5	0/5	ND
ASW suspension							
6.3×10^8	60	ND	1.2×10^2	46.6 ± 4.3	3/3	3/3	3.7×10^3
4.1×10^8	70	ND	ND	43.8 ± 7.2	1/3	3/3	8.9×10^2
2.3×10^8	88	ND	ND	10.5 ± 4.9	0/3	0/3	ND

a. Values give the percentages of green fluorescent cells.
b. *V. cholerae* cells were enumerated by plate count on TCBS agar.
NT, not tested; ND, not detected.

mice were treated with iron dextran, then inoculated intraperitoneally with various numbers of *V. cholerae*, freshly grown in TSB. This resulted in high mortality with an LD_{50} of 1.9×10^3 cfu, and bacteria were recovered from the caecum contents of all the mice tested (Table 1).

Next, *V. cholerae* were collected from the ASW suspension at 60, 70 and 80 days of incubation, and the iron-dextran-treated mice ($n = 3$ for each group) were inoculated intraperitoneally with $2.3–6.3 \times 10^8$ bacterial cells (these doses were 10-fold lower than the LD_{50} of lipopolysaccharides from *V. cholerae* O1 in mouse, data not shown). Three mice inoculated with *V. cholerae* that was incubated in ASW for 60 days died, as did one mouse inoculated with *V. cholerae* incubated in ASW for 70 days. (Sixty and 70 days of incubation were the respective time points at which the bacteria lost culturability on TSA or TSA-SP.) In contrast, all the mice inoculated with *V. cholerae* incubated in ASW for 80 days survived (Table 1).

By direct plating on TCBS agar, *V. cholerae* cells were recovered from the caecum contents of all the mice inoculated with samples that had been incubated for 60 or 70 days. The number of cells recovered was $3.7 \times 10^3 \pm 650$ cfu (60 days) or $8.9 \times 10^2 \pm 330$ cfu (70 days) g^{-1} of gut contents (Table 1). However, no cells were recovered from any of the mice inoculated with bacteria that had been incubated in ASW for 80 days (Table 1). These observations suggested that *V. cholerae* cells in ASW retained sufficient pathogenicity to establish a lethal infection in mice for up to 70 days of incubation in ASW. We therefore defined the VBNC state in this study as bacteria that did not form colonies on TSA or TSA-SP (i.e. 70 day incubation in ASW) but retained their pathogenicity. This criterion reduced the likelihood that the infectious bacteria were injured cells (i.e. unable to grow on TSA, but culturable on TSA-SP) that were rescued by the *in vivo* environment.

Transcriptional characterization of stationary-phase and VBNC-state V. cholerae

As *V. cholerae* cells could be resuscitated from the VBNC state *in vivo*, it was clear that the dormant *Vibrio* cells remained viable and infectious. To investigate the transcriptional activity of VBNC *V. cholerae* cells, we collected the total RNA from bacteria incubated in ASW at 0 (unstressed) or 70 (VBNC) days (the bacterial suspensions were same as is used for mice infection experiment), and the RNAs were labelled with Cy3 or Cy5 and applied to an oligonucleotide-spotted microarray representing about 92.5% (3707/4008 genes) of the *V. cholerae* genome, and differentially expressed genes were identified by statistical analysis of the fluorescence intensity values (Tusher *et al.*, 2001; Merrell *et al.*, 2002).

From the total 3707 genes, those that were induced or repressed by a more than twofold difference between the VBNC and unstressed bacteria were classified by functional group (Fig. 2). The general distribution of genes among the various functional groups in the VBNC state was similar to that obtained for the stationary cells, suggesting the VBNC bacteria may retain the functions essential for viability. However, there was a trend for there to be fewer genes showing the highest levels of expression in the VBNC state than in the stationary phase. This trend was apparent in most 'housekeeping' categories such as protein synthesis (25 stationary versus 15 VBNC), energy metabolism (45 versus 37) and cell envelope (26 versus 20), while the trends seemed to be reversed for the other categories, such as cellular processes (30 versus 46), regulatory function (31 versus 47),

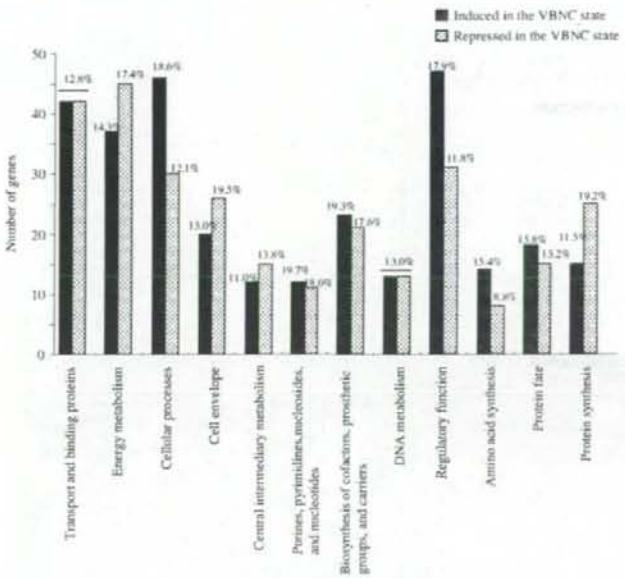

Fig. 2. Functional classes of differentially expressed *V. cholerae* genes. Functional categories of genes showing 2.0-fold or greater changes in expression in the VBNC state compared with unstressed bacteria. The percentage of genes in each category appears above each bar.

amino acid synthesis (8 versus 14) and protein fate (15 versus 18) (Fig. 2 and Supplementary material). This trend could reflect a difference in culturability, an increase in the expression of genes that were poorly expressed in the unstressed condition, the presence of a mixed population of cells in various physiological stages, or a combination of these effects.

We next listed genes showing the most modulation in expression level, using the criterion that there be a fivefold difference in expression between the VBNC and unstressed cells (Fig. 3). Of these, 49% encoded proteins for transport and binding, cellular processes, DNA metabolism, cell envelope, energy metabolism and regulatory functions. In particular, considerable information about the VBNC state was reflected in the altered expression levels of proteins responsible for cellular processes.

Transport and binding proteins. The largest number of genes with induced expression in the VBNC state encoded transport and binding proteins (Figs 2 and 3). In response to environmental stress, *V. cholerae* produces siderophore vibriobactin and a number of metal-binding outer membrane proteins (Tashima *et al.*, 1996). Therefore, the upregulation of genes responsible for the transport of iron (VCA0230, VCA0685, VC2078), magnesium (VC2534), potassium (VCA0194) and cobalamin (VC2381), may account for the protective function of the dormant pathogen. In contrast, two genes (VC2528 and VC1426, which encode, respectively, the ATP-binding ABC-transporter protein and the spermidine/putrescine ABC-transporter membrane component) were repressed. In *Escherichia coli*, such spermidine-preferential proteins have been characterized as part of the polyamine transport system, suggesting that the polyamine uptake system may not function in the survival of the VBNC bacteria.

Cellular processes. Among the 111 genes encoding chemotaxis-related proteins, 25 were induced and 12 repressed in the VBNC state compared with the stationary state (Fig. 2). Among the former genes, some that encoded methyl-accepting chemotaxis proteins (VCA0974, VC2439, VCA0773, VCA1034) were strongly induced in the VBNC state. The role of chemotaxis in the virulence of *V. cholerae* is unclear, but Merrell and colleagues (2002) suggested that motile bacteria exist as non-chemotactic cells in the human host, implying that downregulation of the chemotactic response is linked to dissemination from the host and re-entry into the aquatic environment. The induction of the VC0403 gene [for the MshM, mannose-sensitive haemagglutinin (MshA)-biogenesis protein] in the VBNC bacteria may support this suggestion, because monolayer formation requires the activation of MshA-mediated attachment, accompanied by the transcriptional repression of genes for the flagellum (Moorthy and Watnick, 2004).

In fact, we observed the modulation of duplicate subsets of genes for flagellum-associated proteins: VC2187 (*flaC*, encodes the flagellar-filament assembly component) and VC2132 (*fliG*, encoding a motor protein)

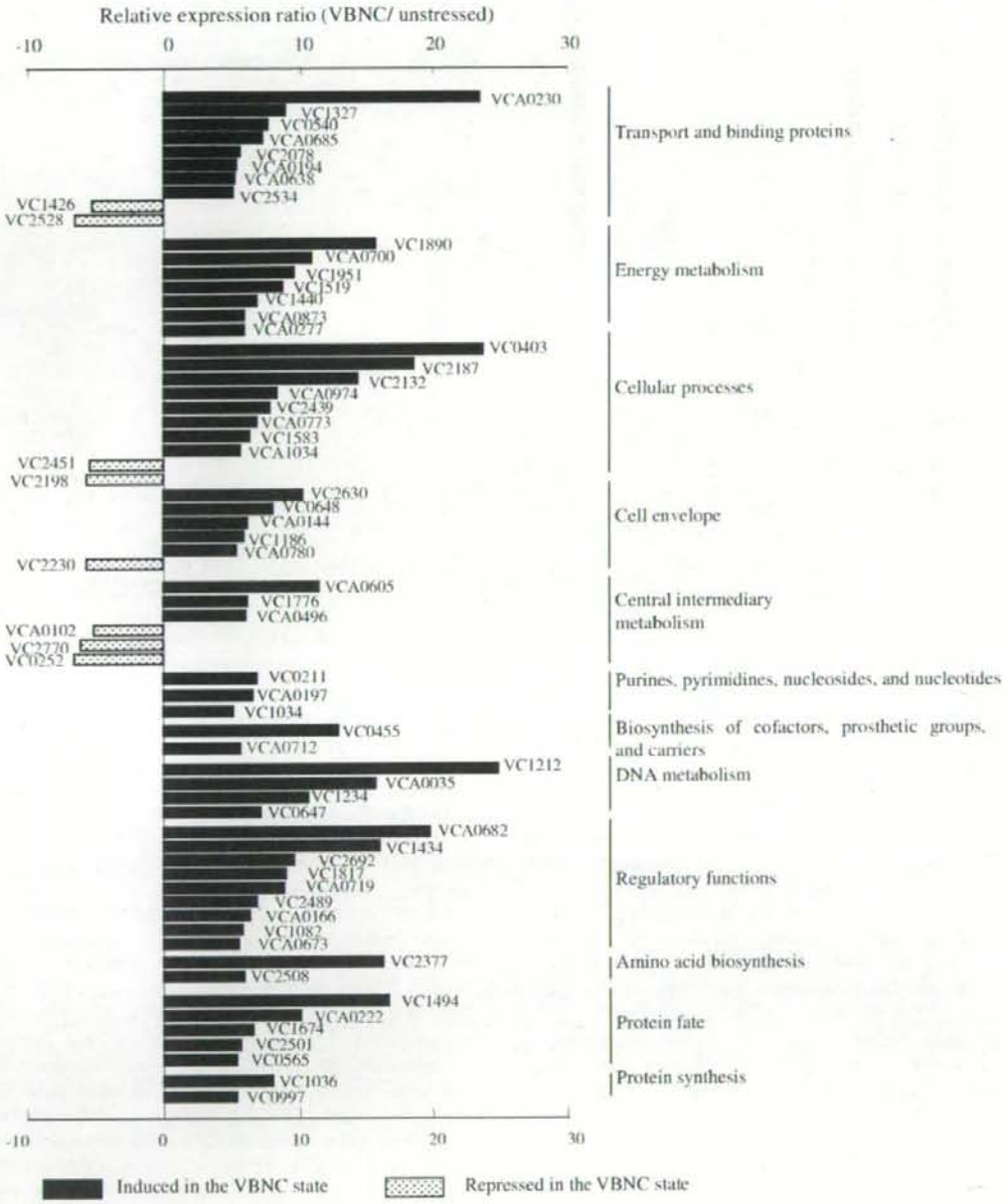

Fig. 3. Gene expression profiles of VBNC *V. cholerae*. Genes showing a 5.0-fold or greater difference between the VBNC state and unstressed bacteria are shown.

were upregulated (18.5- and 14.3-fold), but VC2198 (*flgD*, for a basal body rod modification protein) was repressed as was VC2125 (*fliN*, for a rotor protein), which is essential for flagellar-hook formation (Kutsukake and Doi, 1994) in the VBNC state (Fig. 3).

Under harsh conditions, bacteria sense environmental signals and respond by changing morphologically to take on a coccoid form. This is a major phenotypic sign of stress that is frequently seen in Gram-negative pathogens (Carroll *et al.*, 2001; Alonso *et al.*, 2002; Citterio *et al.*, 2004), and the flagellum is a key feature affecting the morphology, chemotaxis, behaviour and survival of bacteria under these circumstances (Chilcott and Hughes, 2000).

The functional mechanisms of the flagellum are not clear, although a mutation in *flaC* does not affect motility (Klose and Mekalanos, 1998), and a deletion in *fliG* gives a non-flagellate phenotype (Gosink and Hase, 2000). As FliG is a Na+-driven charged protein (Yakushi *et al.*, 2006), the increased expression of *fliG* may result in increased proton pumping to promote osmotic adaptation. Given that flagellar assembly affects biofilm formation (Zhu and Mekalanos, 2003), in which bacterial cells congregate in close proximity, cell–cell communication through the upregulation of these flagellar proteins may improve the survival of the VBNC pathogen in association with biofilm development, quorum sensing and infectivity in *V. cholerae*. Further analysis is necessary to explore the function of this complex machinery in the survival of the bacteria in hostile environments.

In a recent report, Gonzalez-Escalona and colleagues (2006) found that the expression of VC2451 (*relA*) was greater in the VBNC state (induced at 4°C) than in the starvation state (15°C) in *V. cholerae*. The *relA* gene, encoding guanosine pentaphosphate synthase I, is the genetic determinant of the stringent response, because the RelA-catalysed increase in the cellular guanosine nucleotides pppGpp and ppGpp, hereafter referred to as (p)ppGpp, leads to the rapid inhibition of stable RNA biosynthesis, ribosome and protein synthesis, and ultimately, to growth arrest (Cashel *et al.*, 1996). The relatively high expression of *relA* in the VBNC state compared with the starved cells suggests that the VBNC bacteria may be capable of adapting to environmental signals via such stringent responses. In contrast, in this study, we found the expression of the *relA* gene to be 5.4-fold higher in the stationary than in the VBNC cells (Fig. 3). The repression of *relA* in the VBNC state suggests that the intracellular level of (p)ppGpp in the VBNC bacteria was lower than in the unstressed cells, which may also lead to the inhibition of a variety of cellular metabolic events.

DNA metabolism. Among the upregulated genes, except for function-unknown genes, VC1212 (*polB*), encoding DNA polymerase B, showed the greatest increase in expression in the VBNC state (24.8-fold), and the expression of four other DNA metabolism-related genes (VC1897, VCA0035, VC1234, VC0647) was also markedly enhanced (Fig. 3). This finding is consistent with the idea that VBNC cells retain their viability by preserving their DNA metabolism, which is also supported by the observation that 74 of 100 DNA metabolism-related genes were constitutively expressed in the VBNC bacteria (by 0.5–2.0 times the level in stationary bacteria) (Fig. 2).

Cell envelope. Several genes for cell envelope-associated proteins, VC2630 (type IV pilus assembly protein PilQ), VC0648 (lipoprotein NlpL), VCA0659 (ABC-type nitrate/sulfonate/bicarbonate transport outer membrane protein) and VCA0780 (UDP-glucose 6-dehydrogenase), were upregulated in the VBNC state (Fig. 3). Together with MshA, the *pil* gene cluster encodes proteins for the type IV pilus, which confers a significant growth advantage to *V. cholerae* grown on a chitin surface and affects biofilm formation (Meibom *et al.*, 2004; Paranjpye and Strom, 2005).

Energy metabolism. Similarly, VCA0700, encoding chitodextrinase, which plays a major role in the process of chitin utilization in the bacteria, was upregulated in the VBNC cells (Fig. 3). *V. cholerae* can grow even when chitin, an insoluble polymer of GlcNAc, is the sole source of carbon, and several *V. cholerae* proteins with chitinase and chitin-binding activity have been described (Meibom *et al.*, 2004). Among a total of 41 genes for class I chitin-associated proteins (Meibom *et al.*, 2004), VC2217 (beta-N-acetyl-hexosaminidase, *chb2*), VC0048 (*smf*), VC0859 (hypothetical protein) and VC2630 (fimbrial assembly protein, PilQ, described above) showed a more than twofold increase in the VBNC state (*Supplementary material*), suggesting that the dormant bacteria may be capable of recognizing chitin oligosaccharides.

Regulatory functions and others. Among the genes encoding proteins with regulatory functions, 10 genes (VC1696, VCA0682, VC1434, VC2692, VC1817, VCA0719, VC2489, VCA0166, VC1082, VCA0673) showed a more than fivefold upregulation in the VBNC state (Fig. 3). Also, as expected, the virulence determinant and toxin genes continued to be expressed (within a 2.0-fold difference, therefore not shown), indicating the potential pathogenicity of the non-culturable bacteria and in agreement with published findings (Vora *et al.*, 2005).

However, as the genes for the RNA polymerase sigma subunit transcription factors (*rpoA–E*, *rpoH*, *rpoN*, *rpoS*, *rpoZ*) also showed constitutive expression, and as these products regulate the expression of various genes, slight changes in their expression might largely reflect a low

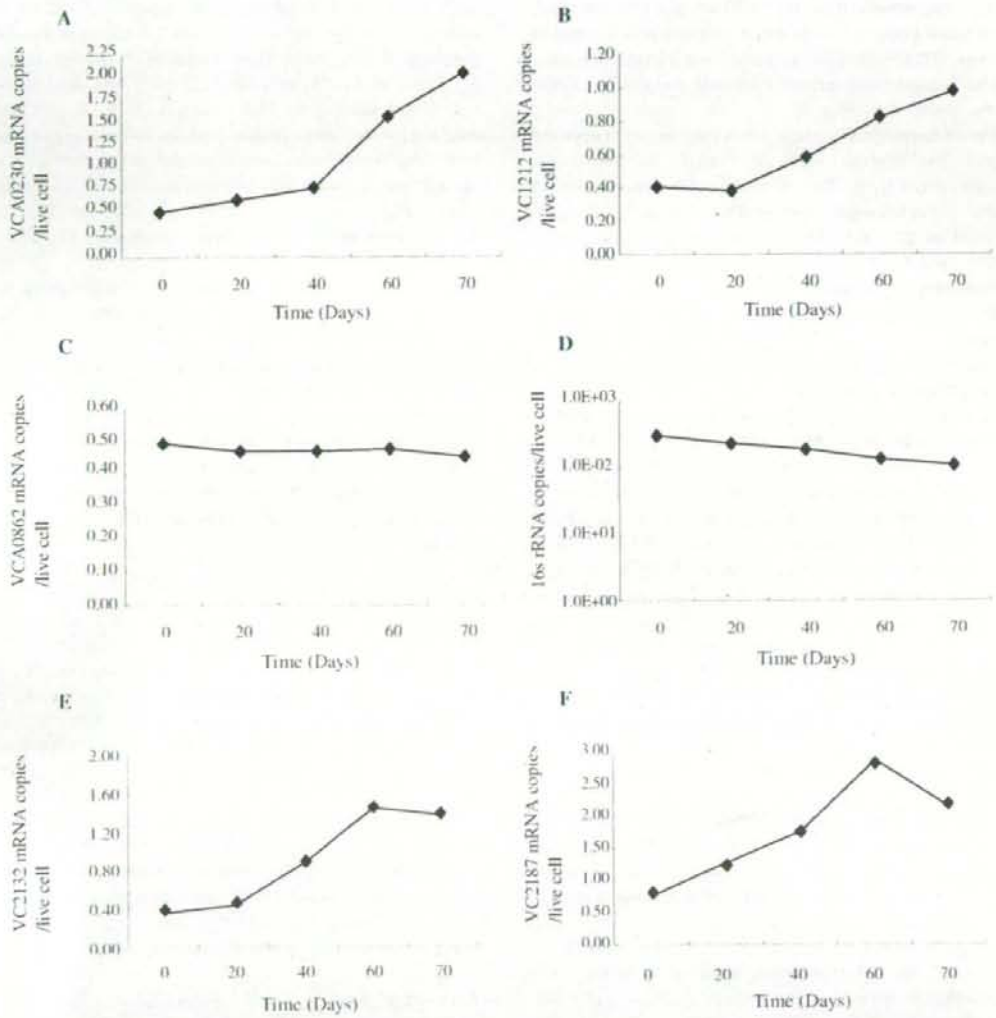

Fig. 4. Abundance of specific RNAs assessed by real-time RT-PCR analysis of *V. cholerae* cells entering and in the VBNC state. (A) VCA0230 mRNA, (B) VC1212 mRNA, (C) VCA0862 mRNA, (D) 16s rRNA, (E) VC2132 mRNA and (F) VC2187 mRNA. Live cells were quantified using BacLight staining as the total number of particles retaining membrane integrity. Bacteria were incubated at 4°C for 0, 20, 40, 60 and 70 days.

copy number of their target genes, as shown in the real-time data (Fig. 4).

In total, 30 genes that continued to be expressed stably in the VBNC state (ratio of 0.995–1.005) were identified (*Supplementary material*). The function of only eight of these genes is known, and we thereafter verified their expression levels in ASW culture by real-time reverse transcription (RT)-PCR.

Real-time RT-PCR monitoring of mRNA expression levels in V. cholerae *in ASW culture*

To verify the microarray data, we performed real-time RT-PCR on RNA prepared from bacteria incubated in ASW for 0, 20, 40, 60 and 70 days, using primers specific for the *V. cholerae* genes VC2132, VC2187, VCA0230, VC1212, VC0862 and VCr001 [encoding FliG, FlaC,

iron(III) ABC transporter, DNA polymerase II, FadL-3 and 16s rRNA respectively). Our array data indicated that the first four genes were highly expressed, whereas the others were constitutively expressed in the VBNC state.

As found with the microarray data, the VC2132 (*fliG*) and VC2187 (*flaC*) mRNA levels gradually increased in response to cold shock, reaching a plateau at 60 days of incubation (either 4.0- or 3.8-fold increases, Fig. 4). VCA0230 and VC1212 also showed increased expression levels, but VCA0230 increased more than the others after the loss of culturability (a 4.1-fold increase at 70 days of incubation, Fig. 4), suggesting that its expression was directly correlated with the timing required for a survival mechanism for the dormant state. Also in agreement with the microarray data, the VCA0862 (*fadL-3*) mRNA level was stable, as was that of VCr001 (16s rRNA) (Fig. 4), which suggests that these genes might be essential for viability and could become valuable genetic markers for the detection and quantification of viable *V. cholerae*. In some cases, the expression levels were lower than one copy per cell (Fig. 4), perhaps because of changes in their gene regulation by central transcriptional or translational factors, or by self-regulation in response to foreign stresses.

In summary, we induced the VBNC state in *V. cholerae* and found the bacteria could be resuscitated *in vivo* and go on to colonize the murine gut of iron-dextran-treated mice. Understanding bacterial gene expression patterns during the VBNC state provides considerable biological information on bacterial physiology, pathogenesis and epidemiology. The gene expression data of *V. cholerae* from human stool (Merrell *et al.*, 2002; Xu *et al.*, 2003) are apparently different from those in the VBNC state. As the significance of the VBNC cells in the environmental transmission of *V. cholerae* to humans is not yet clear, our findings may help identify the process by which cholera epidemics are propagated by dormant bacteria in the aquatic environment. This would help elucidate the ecology of waterborne pathogens and facilitate the assessment of water bacteriological quality, which ultimately could have a great impact on the control of infectious disease transmission.

Experimental procedures

Bacterial strains and cultivation

Vibrio cholerae O1 El Tor strain P6973 was used for this study. Bacteria were grown in tryptic soy broth (Becton Dickinson, MA, USA), and the culturable cells were enumerated by plating on TSA either supplemented with 0.1% sodium pyruvate (TSA-SP plates) (Wako Pure Chemicals, Tokyo, Japan) or without supplement (TSA plates), unless otherwise indicated.

Induction of the VBNC state in V. cholerae

To experimentally generate the VBNC state in *V. cholerae* O1 El Tor strain P6973, approximately 3.7×10^9 cfu of the microorganism grown in TSB for 20 h at 37°C (OD_{600} = 1.60–1.65) were washed with sterile PBS three times and incubated in 200 ml of ASW 24.7 g NaCl, 0.67 g KCl, 1.55 g $CaCl_2 \cdot 2H_2O$, 4.66 g $MgCl_2 \cdot 6H_2O$, 6.29 g $MgSO_4 \cdot 7H_2O$, 0.18 g $NaHCO_3$ per litre at 4°C for up to 120 days. At 10 day intervals, bacterial cell counts were performed by plating 0.1 ml of the suspension on TSA and TSA-SP plates. The membrane integrity was simultaneously tested using the BacLight LIVE/DEAD Bacterial Viability kit (Molecular Probes, Eugene, MA), according to the manufacturer's instruction. This test was performed in triplicate, and the data are indicated as means ± standard deviations (Fig. 1).

Mouse infection assay

Specific pathogen-free female BALB/c mice aged 6–8 weeks (25–30 g) were purchased from CLEA Japan and acclimated for 1 week prior to use. Iron dextran (Sigma) at 250 μg g^{-1} body weight was injected intraperitoneally into the mice with 30 min prior to the bacterial infection, according to the method of Starks and colleagues (2000). The bacteria grown in TSB for 20 h at 37°C were diluted to concentrations of 2.8×10^1–2.1×10^6 cfu with sterile PBS, and mice treated with iron dextran were inoculated intraperitoneally with these cells. Control animals were treated with iron dextran and then inoculated with sterile saline.

The total cell counts of *V. cholerae* collected from ASW suspensions incubated for 60, 70 and 80 days were determined using a cell chamber slide after SYTO9 staining, and approximately 10^8 of the cells were resuspended in 100 μl sterile PBS. Iron-dextran-treated mice were then intraperitoneally inoculated with the cells, as described above. Simultaneously, the cell suspension and its serial dilutions were plated on TSA and TSA-SP to enumerate the cfu. Mouse survival was monitored for 48 h post infection, and mouse motility was scored. For statistical analysis, we tested differences in survival by Student's *t*-test, and the LD_{50} was estimated by the Probit method with $P < 0.05$.

At 48 h post infection, all the mice were sacrificed and 1 g of caecum contents was collected and homogenized with sterile PBS. The homogenate and its serial dilutions were plated on TCBS agar (Eiken Kagaku, Tokyo) to enumerate the bacterial burden in the mice. Representative colonies were subjected to *V. cholerae*-specific PCR using the *ctxA* primer, as described previously (Hoshino *et al.*, 1998).

Microarray experiments

RNA isolation and labelling. Total RNA was isolated using the RiboPure Bacteria RNA isolation kit (Ambion, TX, USA), according to the manufacturer's instructions. After monitoring the stability and quantity of the RNA with a 2100 Bioanalyzer (Agilent), fluorescence-labelled cDNA was synthesized by direct incorporation of Cy3-dUTP or Cy5-dUTP (GE Healthcare Bio-science) during random hexamer-primed reverse transcription, using 20 μg of total RNA and

the CyScribe First-strand cDNA labelling kit (GE Healthcare Bio-Science).

Hybridization and signal detection. Custom oligonucleotide microarrays were obtained from Combimatrix Corp. (Mukilteo, WA, USA). The probes were designed to detect the directly labelled mRNA from 3707 genes on chromosomes I and II of *V. cholerae* O1 biovar El Tor strain N16961 (NCBI accession Nos. NC_002505 and NC_002506, which contain data from 2889 and 1119 genes respectively; data for some of the genes could not be used in the microarrays, because of their short open reading frames). The probes were loaded on a microarray at least in triplicate.

The microarray was pre-hybridized for 1 h at 42°C in a solution containing 5× SSC, 0.1% SDS, 400 mg ml^{-1} bovine serum albumin, and 100 ng µl^{-1} salmon sperm DNA. After being washed three times with double distilled water for 2 min, the microarray was hybridized with heat-denatured labelled cDNA in 5× SSC, 0.1% SDS and 10% formamide for 16 h at 42°C. The array was then washed with 5× SSC and 0.05% SDS for 4 min, with 0.5× SSC for 1 min, and with 2× PBS for 4 min, and fluorescent images of the Cy3 and Cy5 dye channels were obtained using a GenePix 4000B (Axon Instruments, CA, USA).

Data analysis. The signal intensity of each spot and its local background were determined using an Array-Pro Analyzer (Media Cybernetics). The net intensity was calculated by subtracting the mean intensity of all pixels within the local background area from the mean intensity of all pixels within each spot area. Biases in net intensity between the two fluorescent dye channels in a microarray were normalized by locally weighted linear regression analysis (LOWESS normalization) (Yang *et al.*, 2002). Standard ratios of hybridization signals of the VBNC versus stationary DNA probes on each spot were used to represent the expression level of each gene. The average relative expression values of each gene after the adjustment of background values were statistically analysed with a threshold of a 2.0-fold change (for category profiling, see Fig. 2; the detailed data are given in the *Supplementary material*) or 5.0-fold change (for identifying genes induced in the VBNC state, Fig. 3) and a 0% false discovery rate. The raw data are also available at ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) under accession number A-MEPX-581.

Real-time RT-PCR

Bacterial total RNA was isolated from *V. cholerae* cells incubated in ASW for 0, 20, 40, 60 and 70 days, as described above. cDNA was synthesized from 1 µg of RNA using a RetroScript cDNA synthesis kit (Ambion), according to the manufacturer's instructions. The cDNA levels of all the genes were determined by quantitative RT-PCR in 50 µl reaction volumes, with the following conditions: 5 µl synthesized cDNA solution, 25 µl 2× Power SYBR master mix (Applied Biosystems), 0.9 µM of each forward and reverse primer in an ABI Prism 7500 Sequence Detection System (Applied Biosystems). Threshold cycle values were calculated from the amplification plots, and the gene expression levels were compared with the amplification data obtained from genomic DNA. Gene-specific primer pairs were designed using Primer Express ver. 3.0 (Applied Biosystems): VC1212 (5′-TTCGCCATTCAGCGTTCAC-3′ and 5′-CACGAGCCACCAATGC-3′), VCA0230 (5′-CGTGAGTTGGTGGATGGA-3′ and 5′-GACCAACACCGCTAGCTTT-3′), VCA0862 (5′-GCCGCCAAAGCCAAATT-3′ and 5′-CCATGAAGGGAGCCAATCC-3′), VCr001 (5′-CGGTAATACGGAGGGTGCAA-3′ and 5′-CACCTGCATGCGCTTTACG-3′), VC2132 (5′-CTGGCCGAGTTGAACGAAAT-3′ and 5′-TGTGCCCCCGCTTA-3′), and VC2187 (5′-GAGAGTGTGGCGATTGTCGAT-3′ and 5′-GAGTTCAGCACATGCTATCC-3′). Duplicate sets of experiment were performed and the standardized data was shown in Fig. 4.

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