Cloning and Characterization of Two Types of $tonB$ Genes, $tonB1$ and $tonB2$, and Ferric Uptake Regulator Gene, $fur$ from Photobacterium damselae subsp. piscicida

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ABSTRACT—There is some evidence that the virulence of some pathogenic bacteria is related to their iron uptake system. To better understand this system, three genes involved in iron uptake of Photobacterium damselae subsp. piscicida ($tonB1$, $tonB2$ and iron uptake regulator or $fur$) were cloned from a genomic library and sequenced. The amino acid sequence identities of $tonB1$ and $tonB2$ with the corresponding sequences of Vibrio spp. range from 32.4 to 39.5% and from 43.6 to 50.5%, respectively. The amino acid sequence identities of $fur$ with the corresponding sequences of other Vibrio spp. are over 85%. We were able to construct a $fur$ gene knock-out mutant using the marker exchange method. Using this knock-out mutant, we showed that $tonB1$, but not $tonB2$, is regulated by $fur$ in P. damselae subsp. piscicida. The $fur$ gene is also important for iron regulation since it has an influence on the expression of the heme receptor gene, $hutA$. The $fur$ gene knock-out mutant generated in this study will be helpful in future studies of determining the role of iron in P. damselae subsp. piscicida pathogenesis and virulence.

Key words: Photobacterium damselae subsp. piscicida, iron acquisition, TonB, ferric up take regulator, fur, tonB

Pasteurellosis is an economically important disease that leads to severe losses in farmed yellowtail Seriola quinqueradiata in Japan (Egusa, 1983), and some farmed marine fishes in the Mediterranean, and farmed and wild marine fishes in the United States. (Toranzo et al., 1991). It is caused by a halophilic bacterium Photobacterium damselae subsp. piscicida (formerly known as Pasteurella piscicida). The ability of pathogenic bacteria to acquire iron is essential both for their growth and pathogenicity. In the presence of iron, the host has an increased susceptibility towards P. damselae subsp. piscicida, apparently since an elevated iron level of concentration increases virulence, inhibits some non-specific immune defense processes or a combination of both (Magariños et al., 1994). Inside the host, the amount of free iron available to invading bacteria is limited, because intracellularly most iron is in the form of heme and extracellularly bound to high-affinity carrier proteins such as transferrin and lactoferrin (Byers and Arceneaux, 1998). As a consequence, pathogens have developed effective iron acquisition mechanisms (Henderson and Payne, 1993; Payne, 1994; Braun and Killmann, 1999) to enable them to sequester the required amount necessary for their survival and growth. In addition to these acquisition mechanisms, Gram-negative bacteria express three proteins (TonB, ExbB and ExbD) that are required for high affinity iron transport systems (Seliger et al., 2001). In Vibrio cholerae, heme utilization involves the interaction of the genes $hutA$, $tonB1$, $exbB1D1$, $hutB$ and $hutCD$ (O'Malley et al., 1999), although the recently identified genes, $tonB2$ and $exbB2D2$ may have the same function (Occhino et al., 1998).

The function of the TonB system is to couple the energy from the cytoplasmic membrane to the outer membrane receptor proteins (Postle, 1993). Stabilization of such reaction in the inner membrane requires ExbB and ExbD (Ahmer et al., 1995; Higgs et al., 1998). TonB is thought to be interacted with the outer membrane receptor after the ligand has bound to the

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Accession number: $tonB1$ and $exbB1$: AB105427; $exbD$ and $tonB2$: AB105428; fur: AB070654
receptor. Such interaction leads to a conformational change in the receptor which mediates transport of the ligand through the receptor pore (Letain and Postle, 1997). However, there are instances when the availability of iron reaches extremely high levels rendering it also toxic to pathogens. This toxicity is due to the ability of iron to catalyze Fenton reactions and the formation of reactive oxygen species (Escolar et al., 1999). Iron uptake in bacterial systems, therefore, must be regulated to maintain the intracellular concentration of the metal within desirable limits.

Much has been learned about regulation of iron transport in bacteria from Escherichia coli mutant, fur (ferric uptake regulation), that constitutively expresses all genes that are known to be inhibited by iron (Hantke, 1981; Escolar et al., 1999). The fur gene has been mapped (Bagg and Neilands, 1985), cloned (Hantke, 1984) and sequenced (Schaffer et al., 1985), and some basic aspects of its regulation mechanism have been elucidated (Hantke, 1981, 1984).

P. damselae subsp. piscicida may be exposed to different environmental conditions because it infects diverse fish species. P. damselae subsp. piscicida may obtain iron by producing siderophores or may have evolved other iron-acquisition mechanisms not related to siderophore production (Bakopoulos et al., 1997). Naka et al. (2002) sequenced part of the genomic DNA of P. damselae subsp. piscicida to elucidate the different genes and the corresponding mechanisms involved in its virulence. In order to identify the genes and mechanisms involved in the iron acquisition of P. damselae subsp. piscicida, we cloned and characterized its TonB system. Juiz-Rio et al. (2004) recently reported the nucleotide sequence of the fur gene in P. damselae subsp. piscicida strain D121. We constructed a P. damselae subsp. piscicida fur knock-out mutant by the marker exchange method in order to analyze the role of the fur gene and its relationship with the TonB system in the iron acquisition mechanism.

Materials and Methods

Bacterial strains, plasmids and culture media

P. damselae subsp. piscicida P 97-008 strain was isolated from yellowtail at the Nagasaki Prefecture, Japan in 1997. It was grown in either brain heart infusion broth (DIFCO, USA) containing 2% NaCl (2BHI) or in 2BHI containing 1.5% agar (DIFCO, USA) at 25°C. Trypticase soy broth (DIFCO, USA) containing 2% NaCl (TSBS) was used for the culture for RT-PCR analysis. Escherichia coli strains were cultured in 2x YT broth or in 2x YT agar at 37°C. E. coli strains carrying the plasmid pRE112 (Edwards et al., 1998) were cultured in 2x YT medium supplemented with the appropriate antibiotics. pUC119 was used as a clonig vector.

Cloning of tonB1 and tonB2 genes

Partial fragments of the tonB1 and tonB2 were obtained from sequencing of the P. damselae subsp. piscicida genomic library (Naka et al., 2002). These were used as probes to obtain the full length sequences of the genes. Plasmids were extracted from the positive colonies by the alkaline lysis method (Sambrook and Russell, 2001), and sequenced using an automated sequencer (Li-Cor, USA). The DNA and predicted amino acid sequences were analyzed using the GENETYX computer program (SDC software Development, Co., Japan).

Cloning of the ferric uptake regulator (fur) gene

P. damselae subsp. piscicida fur gene was PCR-amplified using degenerate primers (furF: ATGTCAAGA- YAATAAYCARGC and furR: TTNCCNCTTCAAAR- TGGTG) designed from sequences of previously reported fur genes of several Vibrio species. Amplification conditions were as follows: an initial denaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 1 min, and a final elongation at 72°C for 5 min.

The amplified fragment was purified and was subsequently used as a probe to obtain the full length sequence of the fur gene from the genomic library. Plasmids from the positive colonies were extracted and sequenced as described previously.

Construction of the fur gene knock-out

The kanamycin-resistant gene from pp-kan (Kim and Aoki, 1994) was inserted into a BamHI site in the ORF of the fur. The fragment containing the pp-kan was ligated into a suicide vector, pRE112 (Edwards et al., 1998), then transformed into E. coli SM10 (Simon et al., 1983), and finally transferred by conjugation to P. damselae subsp. piscicida P97-008sm, a streptomycin-resistant strain. The transconjugants were streaked on BHI agar supplemented with 2% NaCl, 20 µg/mL of kanamycin and 1,000 µg/mL of streptomycin. The resistant strain was cultured in drug-free 2BHI and plated on 2BHI agar containing 20 µg/mL kanamycin, 1,000 µg/mL of streptomycin and 5% sucrose to select for strains that had undergone a second recombination event.

Characterization of tonB genes

To determine the role of the fur gene in regulating tonB1 and tonB2 activity, the mutant P. damselae subsp. piscicida strain carrying the fur gene knock-out and a wild-type P. damselae subsp. piscicida were cultured in parallel. Overnight cultures of P. damselae subsp. piscicida in TSB broth were supplemented with either FeSO₄ to a final concentration of 10 mM or 2,2'-dipyridyl, an iron chelator, to a final concentration of 100 µM or
tonB genes of *P. damselae* subsp. *piscicida*

300 μM. After addition of the chemicals, they were further incubated for an additional 8 h at 25℃. Bacterial culture with no chemicals added served as a control.

After the desired incubation time, the bacterial cultures were centrifuged at 4,000 x g for 10 min. Bacterial total RNA was extracted with Trizol (Invitrogen, USA) following the manufacturer’s protocol. Total RNA (1 μg) was treated with DNase (Promega, USA) for 1 h, and reverse-transcribed to its cDNA using a reverse transcription kit (ReverTra Ace α, Toyobo, Japan).

*P. damselae* subsp. *piscicida* tonB1, tonB2, heme receptor (pphutA) and fur genes were PCR-amplified using their corresponding sets of primers. One microliter of cDNA template was used in a 30 μL reaction. PCR conditions were the same as described previously except for the amplification of 16S rRNA gene which was conducted for 25 cycles.

**Results**

Nucleotide and putative amino acid sequences of tonB1 and tonB2 genes from *P. damselae* *subsp.* *piscicida*

The nucleotide and putative amino acid sequences of the *P. damselae* subsp. *piscicida* tonB1, designated as *pptonB1*, is shown in Fig. 1. The *pptonB1* gene consists of 756 bp coding for 252 amino acids. Another

Fig. 1. Nucleotide and putative amino acid sequences of *pptonB1* and partial *exbB1* genes from *P. damselae* subsp. *piscicida*
Fig. 2. Nucleotide and putative amino acid sequences of pptonB2 and partial exbD2 genes from P. damselae subsp. piscicida.

Fig. 3. Alignment of pTonB1 with comparison of those of three other bacteria. Identical amino acid residues are indicated by the dot (.).

PPB1  1 MDTRYVIAGAVSVIIHGILSAVPSQNAIAMPI—GNQIAPVSLPNLVTAPIAPPQPD  58
VCB1  1 .N.LN. . . . GL.LAF.A.L.ITTDEAQVF—.—..PTQ.S.I.M.N.M.KVA.AQEPQ  58
VPB1  1 .NVV. . . . GA.LWV.A.AL.FVSQENKVF—.—..PASS.S.S.S.PDTLEKPT  58
VVPB1 1 .—.—.—.—.—M.L.AA.PE.RQ.—VIFQTNA.SDS.S.S.S.SN.P. —.—.—.—.—SSN-N  41

PPB1  59 VAKAEVKQPQSVKETQKFQIEQRTA—VK—KLVEKTTPIPAAKVKVAKNNKTTPK  114
VCB1  59 TQETESKLEVSFKT—PVQ——E.V—KTPPA.FKEVH.P.KP.TQP.KPQ.EQ.—VS  108
VPB1  59 BNVUPEPE.—PKQPQAGKKVCKVQ.—K.EAV.KKVE.KPEPQPKPKE—.QTA—  115
VVB1  42 V.E.N.—.E.KEBT.FPKPKP.—.EPK.E.FPK.TTPKASLEKPVVEN.SST—.A  98

PPB1  115 VKNRDERPVSATKPKQPKNTQSVSVNTNKKALLSSQTVSPELVSRPTAPTRPGP  174
VCB1  109 K-SVAE—.OQKEVTAEMAK.PAPIPOQPSQPTA.SQGITS.PILVDK.—ALVSAQQV  165
VPB1  116 KRE—.—.K.AEPPKIEPK.DTVT-T.—Q.ASASQSGA-TS.PILVDK.—VSQ.TQ  168
VVB1  99 ———.EST—.EQEPFPEVHKT—.GADNIPASSQGVS——.S-T.EK.S.MEK  148

PPB1  175 VSYPKLIHERKQIQVGMVEIWPDKPKQIKQIQ-IVSSQGELDAKAIKKWEFSSHII  233
VCB1  166 PR.I.RK.—.ET.Y.LAQN.QU.LL.S.GEIA—.S.E.Q.K.P.  224
VPB1  169 PR.S.Q.—B.ALYVLE.EN.HVLE.GTES.—AS.R.Q.Q.TP.  227
VVB1  149 FV. —B.TLAFI.LQ.LQ.LQ.LQ.LR.AM.—.D.R.—Q.K.TP.  207

PPB1  234 VDGQAIHRVPFVPFQD—  252
VCB1  225 L.VPV. . . .I.I.E.KG  244
VPB1  228 LG.LKV. . . . . K.EG  247
VVB1  208 EN.KMPS. —.R. —.S.  226
gene, exbB1, is located downstream of pptonB1. On the other hand, tonB2, designated as pptonB2, consists of 654 bp coding for 218 amino acids (Fig. 2). Fig. 3 shows an amino acid alignment comparison of pptonB1 with TonBs from other bacterial species. pptonB1 has 40%, 40% and 36% amino acid identity with TonBs from Vibrio cholerae, V. parahaemolyticus, and V. vulnificus, respectively. On the other hand, the respective amino acid identities of pptonB2 with previously reported TonB2s were 49%, 50% and 46% (Fig. 4).

Nucleotide and putative amino acid sequences of the P. damselae subsp. piscicida fur

The P. damselae subsp. piscicida fur is composed of 444 bp which codes 148 amino acids. This amino acid sequence is almost 100% identical to the one already reported by Juiz-Rio et al. (2004). The P. damselae subsp. piscicida Fur protein we are reporting in this manuscript possesses a different residue at position number 6 of the mature protein, being in our case a Gln instead of His.

Expression of pptonB1, pptonB2 and pphutA in the wild type and in a fur gene knock-out mutant was analyzed under a normal, an iron-rich and iron-limiting conditions (Fig. 5). pptonB1 was not detected in the wild-type P. damselae subsp. piscicida under the iron-rich condition, but an increasing level of expression was observed under the iron-limiting conditions. On the other hand, expression of pptonB1 in the mutant strain was detectable under the iron-rich condition, and high under the iron-limiting conditions. In contrast, pptonB2 was slightly expressed in both wild and mutant types, regardless of the culture conditions. The heme receptor gene, pphutA, in the wild type was weakly expressed under the iron-rich condition but elevated in the iron-limiting conditions. However, in the fur gene knock-out mutant strain, the level of pphutA expression under the iron-rich condition was apparently high. The levels of 16S rRNA expression in both strains (wild type and mutant) were not significantly different because the intensities of the bands were indistinguishable between the culture conditions.

Characterization of tonB1, tonB2 and pphutA expression

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Discussion

We were able to identify two types of tonB genes, the tonB1 and tonB2, in P. damselae subsp. piscicida. For several years the tonB gene was reported to be present as a single copy. Two types of tonB genes have been described in V. cholerae (Occhino et al., 1998) and in P. aeruginosa (Zhao and Poole, 2000). V. parahaemolyticus and V. alginolyticus also have sequences homologous to tonB1 and tonB2 (O'Malley et al., 1999) and may be present in Neisseria (Desai et al., 2000). Seliger et al. (2001) found that the V. cholerae tonB genes have unique as well as common features. Both TonBs can mediate transport of hemin and the siderophores vibriobactin and ferrichrome. TonB1 mediates utilization of the siderophore schizokenin, while TonB2 is required for the utilization of enterobactin. Seliger et al. (2001) showed that the two TonB proteins differ in amino acid sequence and protein size. It is believed that the existence of two types of tonB genes would be useful for pathogenic bacteria to adjust its iron metabolism towards changes in the environment (Seliger et al., 2001). Sequence analysis of the P. damselae subsp. piscicida tonB genes showed that the tonB1 exceeds tonB2 by almost 100 base pairs. V. cholerae TonB1 is 38 amino acids longer than the TonB2 and this was probably responsible for its superior ability to function at higher osmolarity. In the case of P. damselae subsp. piscicida, further studies are needed to determine their significance to specific physiological mechanisms in the bacterial system.

We have also cloned the P. damselae subsp. piscicida fur gene and identified its putative promoter sequence. A strain containing the fur knock-out mutant was successfully constructed using the marker exchange method. fur knock-out mutants have been previously constructed in V. vulnificus (Litwin and Calderwood, 1993b) and in V. cholerae (Litwin and Calderwood, 1994) using the same method. Lam et al. (1994) also developed fur mutations in V. cholerae using manganese mutagenesis. Many studies have clearly demonstrated that fur also functions in some cellular processes not directly related to iron metabolism, including acid shock response (Hall and Foster, 1996), defense against oxygen radicals (Niederhofer et al., 1990; Tardat and Touati, 1993), chemotaxis (Karjalainen et al., 1991), metabolic pathways (Hantke, 1987; Stojilkovic et al., 1994), bioluminescence (Makemson and Hastings, 1982), swarming (McCarter and Silverman, 1989), and the production of toxins and virulence factors (Litwin and Calderwood, 1993a). The establishment of a fur knock-out mutant should pave the way to a better understanding on the possible role of the fur gene in a particular bacterial species.

Our results using the P. damselae subsp. piscicida fur knock-out mutant suggest that fur is involved in iron regulation since the heme receptor, hutA is regulated by the fur gene. Moreover, it was also observed that tonB1 but not tonB2 is regulated by the Fur protein. This has possible implications on the iron acquisition mechanism of P. damselae subsp piscicida, in which tonB1 may be directly involved in iron acquisition and tonB2 may have other roles in the cellular process which are different from or may complement the iron uptake mechanism. The presumed varying functions of the two TonBs of P. damselae subsp. piscicida have implications with respect to its survival in different environments.

Our results show that a fur knock-out mutant has provided initial information on the iron acquisition system in P. damselae subsp. piscicida. Bakopoulos et al. (1997) demonstrated that P. damselae subsp. piscicida produces elevated levels of high molecular weight products under iron-limiting conditions. We will conduct microarray analysis or 2-dimentional electrophoresis using samples obtained from the wild type and the fur mutant strains in order to identify genes which are regulated by iron concentration in a Fur dependent manner. The results obtained from these experiments will lead us to understand the mechanism of virulence of P. damselae subsp. piscicida. We are planning to generate a tonB1/tonB2 double knock-out mutant to better elucidate the individual of these two genes and to determine whether they have related or independently roles in P. damselae subsp. piscicida virulence.

References


