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CLONING AND SEQUENCING OF THE *FUR* GENE FROM MARINE PROBIOTIC *ALTEROMONAS AURANTIA* A18 AND ITS POSSIBLE ROLE IN SIDEROPHORE PRODUCTION

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ABSTRACT

The complete *fur* gene from the marine probiotic *Alteromonas aurantia* A18 was amplified by PCR and the cloned gene was sequenced. Results indicated that the similarity between the sequence of the *fur* gene from strain A18 and that from *Alteromonas* sp O-7 was 97%. Fur protein of strain A18 was 98% identical to that of *Alteromonas* sp. O-7 and 70% identical to that of *E. coli*. The putative *fur* open reading frame coded for 148 amino acids representing a protein of 16.637 kDa. The recombinant expression vector pQE30-fur was constructed to confirm function of the cloned *fur* gene in *E. coli*. We found that siderophore yields of the transformants bearing pQE30-fur (induced by IPTG) were much lower than those of the parental strain and the same transformants that were not induced by IPTG in the iron-rich medium. The results suggest that some enzymes responsible for biosynthesis and secretion of siderophores in *E. coli* were repressed by the Fur protein encoded by the *fur* gene from *A. aurantia* A18.

Keywords: *Fur* gene, marine probiotic bacteria, siderophore, gene cloning

INTRODUCTION

In the past ten years, mariculture industry in China has been developing very rapidly. However, disease outbreaks are being increasingly recognized as a significant limitation on mariculture production in this country. Most of bacterial infections in marine animals were found to be caused by vibrios. So far, conventional approaches, such as the use of disinfectants and antimicrobial drugs had limited success in the prevention or cure of the bacterial diseases in marine animals. Furthermore, there is a growing concern about the abuse of antimicrobial drugs in mariculture. The massive use of antimicrobial drugs for bacterial disease control in marine animals increases the selective pressure exerted on the microbial population and encourages the natural emergence of bacterial resistance and the horizontal transfer of resistance genes in different organisms. The best alternative strategies to antimicrobial drugs in disease control would be the use of probiotic bacteria as vaccines or microbial control agents (Verschuere *et al*. 2000).

Iron is an essential nutrient for marine bacteria which is difficult to obtain due to its low solubility under the physiological conditions. Most of marine bacteria usually obtain iron by means of siderophore, which introduces iron into the bacterial cells by a specific cell membrane receptor (Li and Chi 2004). However, an excess of iron in the cells is toxic because of its ability to catalyze Fenton reactions and formation of active species of oxygen (Escolar *et al.* 1999).Therefore, iron uptake has to be exquisitely regulated to maintain the intracellular concentration of iron between desirable limits. It was found that Fur (ferric uptake regulate) protein encoded by *fur* gene in marine bacteria is involved in this regulation. The protein acts as a transcriptional repressor of iron-regulated promoters by virtue of its Fe^{2+}-dependent DNA binding activity. There are two configurations of Fur protein in an equilibrium which is displaced by Fe^{2+} towards the form competent for binding DNA and thus for repression of transcription. The lack of iron results in derepression of an entire collection of genes for biosynthesis and transport of siderophore (Escolar *et al.* 1999).

In the previous studies (Wang *et al.* 2002) we found that the marine probiotic A18 can be successfully applied to control the bacterial diseases in marine fish and improve the quality of rearing seawater. The probiotic strain A18 was identified and found to be *Alteromonas aurantia* (Li *et al.* 2001). In another study (Li *et al.* 2008) we found that a mutant of *Alteromonas aurantia* A18 can produce siderophores with hydroxamate group, which are related to the strong inhibition of the pathogenic bacteria. In order to elucidate the regulation mechanisms of the Fur protein in the probiotic strain A18, the *fur* gene from strain A18 was cloned, sequenced and expressed in *E. coli* in the present study.

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MATERIALS AND METHODS

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1.

Growth media and cell cultivation

The marine *Alteromonas aurantia* A18 was cultivated at 27°C in marine broth 2216E and *E. coli* was grown at 37°C on LB medium with or without 50 $mgml^{-1}$ of Ampicilin or/and Kanamycin.

Isolation of total genomic DNA and cloning the *fur* gene from A*lteromonas aurantia* A18 by PCR

The total genomic DNA was isolated from strain A18 according the methods described by Sambrook *et al.* (1989) and was used as the template for cloning of the *fu*r gene by using PCR techniques. The primers of *fur* gene of *A. aurantia* A18 were designed by the homologous clone strategy according to the strain of *Alteromonas* sp. O-7[8] for PCR were as follows: the forward primer was 5'-TGAAATG-ACAGACCACAACTTAGAGC-3' and the reverse primer was 5'-TGGCGAAGTGGAAGGCTAGTTAC-3. The conditions for PCR amplification were as follows: initial denaturation at 94°C for 5 min, denaturation at 94°C for 1 min, annealing temperature at 50 °C for 1 min, extension at 72°C for 1 min, final extension at 72°C for 10 min. The PCR was run for 30 cycles. 0.25 ml of TaKaRa Ex Taq ($5U \cdot ml^{-1}$) was used as DNA polymerase. PCR cycler was GeneAmp^{Ò} PCR System 2400 made by PERKIN ELMER. The size of PCR product was expected to be 0.5kb. The PCR product was electrophoresed on a 0.6% agarose gel.

Table 1. Bacterial strains and plasmids

Strains and plasmids	Genotypes	Sources
Strains		
Alteromonas aurantia A18	Not known	Laboratory (Qingdao University of Science & Technology)
E.coli JM109	*recA endA1 gyrA96 thi hsdR17 supE44 relA1Δ (lac-proAB) /F[traD36 proAB+ lacIq lacZΔM15]*	-do-
E.coli QC1732	F-*Δ*(*argF–lac*)*U169 rpsL Δfur::kan*	CNRS, Marseilles, France
Plasmids		
pUCm-T vector	Amp^r *lacZ*'	Sangon, Shanghai
pGEM T-easy	Amp^r *lacZ*'	Promega (USA)
pQE30	Amp^r *lacZ*'	Qiagen, UK
PUC-fur	Amp^r *lacZ*' pUCm-T with the *fur* gene	This study
pGEM-fur	Amp^r *lacZ*' pGEM with the *fur* gene	This study
pQE30-fur	Amp^r *lacZ*' pQE 30 with the *fur* gene	This study

DNA ligation and transformation

The amplified DNA fragment with 0.5 kb by PCR was excised and purified from the gel by using PCR Fragment Recovery Kit (Shanghai Bioasia Biotechnology CO.LTD) according to the procedures offered by the manufacturer. The DNA fragment was ligated into pUCm-T Vector by using T_4 DNA ligase at 16°C for 16 h and was transformed into the competent cells of *E. coli* JM109. The transformants with pUCm-T carrying the *fur* gene was selected on LB plate with Ampicilin.

Identification of the *fur* gene on the plasmids

The transformants were cultivated in liquid LB medium with Ampicilin at 37°C for 12-16 h. The plasmids were isolated from the cultivated transformant cells according to the methods described by Sambrook *et al.* (1989). The *fur* gene was amplified by PCR using the isolated plasmid as the template. The plasmid with the *fur* gene was named pUC-fur.

Sequencing of the *fur* gene

Sequencing of the *fur* gene on the pUC-fur was carried out by the Shanghai Bioasia Biotechnology CO. LTD.

Alignment of the *fur* genes

Sequences of the fur gene determined in this study were aligned with available fur gene sequences form GenBank such as *fur* genes of *Alteromonas* sp 0-7 (Tsujibo, *et al.* 2000), *E. coli* (Escolar *et al.* 1998), *S. oneidensis* MR-1 (Heidelberg , *et al.* 2002), *Y. pestis* (Staggs and Perry. 1992), (Tolmasky *et al.* 1994), (Litwin *et al.* 1992), (Litwin and Calderwood, 1993), (Stover *et al.* 2000), (Schaffer *et al.* 1985).The phylogenetic tree was constructed by using DNAstar software package.

Expression of the *fur* gene in *E. coli* QC1732

The fur gene was amplified by PCR using pUC-fur as template. The primers for PCR were as follows: the forward primer was 5'-CGGGATCC(*Bam*H I) ATGACAGA CCACAAC-3' and the reverse primer was 5'-CCCAAGCTT(*Hind* III) CTACTCA CCATTTTC-3'. The conditions for PCR amplification were the same as those described above. The PCR products were ligated into pGEM T-easy by

using T_4 DNA ligase at 16°C for 16 h and transformed into the competent cells of *E. coli* JM109. The transformants obtained were cultivated aerobically in liquid LB medium with 50 mg·ml^{-1} of Ampicilin at 37°C for 12 h. The pGEM-*fur* obtained from the transformants and pQE30 (the expression vector from Qiagen) were digested with *Bam*H I and *Hind* III at 37°C for 20 h, respectively. The *fur* gene fragment and the digested plasmid pQE30 were separated on gel by electrophoresis and recovered from the gels by using PCR Fragment Recovery Kit (Shanghai Bioasia Biotechnology CO., LTD) according to the procedures offered by the manufacturer, respectively. The *fur* gene fragments were ligated with the digested plasmid pQE30 by using T4 DNA ligase and transformed into the competent cells of a *fur* null mutant strain *E. coli* QC1732 (Urs A *et al.* 1999). *E. coli* QC1732 (pQE30-fur) obtained were grown on LB plate with 50 mg·ml^{-1} of Ampicilin and Kanamycin at 37°C overnight and the *fur* gene on pQE30 in *E. coli* QC1732 (pQE30-fur) was confirmed by using the same methods described above. The plasmid obtained from the positive transformants was named pQE30-fur. Expression of the *fur* gene in the transformants was induced in liquid LB medium with 1mmol·l^{-1} of IPTG and 50 mg·ml^{-1} of Kanamycin for 10h.

Determination of siderophore

The non-specific assay of siderophore in the supernatants of the culture was carried out using the modified CAS method described by Schwyn *et al.* 1987. The amount of siderophore in the solution was calculated according to the following equation:

$$\text{The amount of siderophore(\%)} = \frac{Ar - A}{Ar} \times 100\%$$

A is OD_{630nm} value of the reaction mixture of the supernatant of the culture and the same volume of CAS solution. The OD_{630nm} value of the liquid LB medium was used as a blank. Ar is OD_{630nm} value of the reaction mixture of the liquid LB medium and the same volume of CAS solution.

Statistical methods

Results of the amount of siderophore production between induced and uninduced *fur* expressions were compared by repeated measures ANOVA. Results are expressed as means ± SD. Differences with a value of $P < 0.05$ were considered significant Carlos H *et al.* 2002).

RESULTS

Amplification of the *fur* gene by PCR

When the total genomic DNA from strain A18 and the primers were used for PCR amplification, only one single band with about 500 bp was obtained in the PCR products (Figure 1).

Sequencing of the PCR products

After purification of the PCR products from the gel, the PCR products were ligated into plasmid pUCm-T and transformed into the competent cells of *E. coli* JM109. The positive transformants on LB plate including Amp, X-gal and IPTG were selected and the plasmids in the transformants were isolated. After the *fur* gene on the plasmid was confirmed by PCR (Figure 2) the *fur* gene was sequenced. The whole nucleotide sequence of the *fur* gene is shown in Figure 3.

Analysis of the Fur proteins

Amino acid sequence of the Fur protein deduced from the sequence of the *fur* gene in strain A18 is shown in Figure 4.

This results showed that the putative fur open reading frame encoded 148 amino acids with molecular mass of 16.637kDa. The homology analysis proves that strain A18 was closely related to Alteromonas sp 0-7.

Involvement of the *fur* gene in siderophore biosynthesis in *E. coli* QC1732

Figur 5 shows the analysis of the PCR products from the expression vector containing the fur gene (PQ 30-FUR). The positive clones were used for the experiments to determine the amount of siderophores.

The amount of siderophores in the cultures used in this experiment are given in table 2. Results have shown that the QC1732 (PQE30fur) when induced to express Fur protein in iron rich medium has resulted in significantly low amount of siderophores.

Tsujibo *et al.* (2000) observed that a serine protease-encoding gene of marine *Alteromonas* sp. O-7 is regulated by the Fur protein. However, it is still unknown whether the Fur protein is related with siderophore biosynthesis and secretion in the marine *Alteromonas* sp. O-7.

DISCUSSION

One single band with about 500bp obtained from PCR amplification reactions (Figure 1) indicated

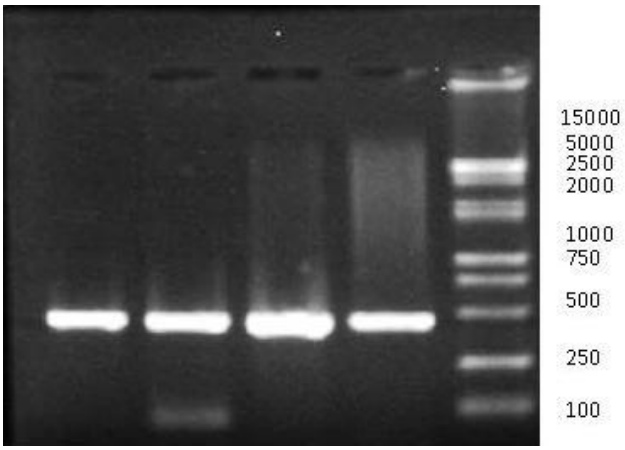

Figure 1. Agarose gel electrophoresis of PCR products amplified from strain A18

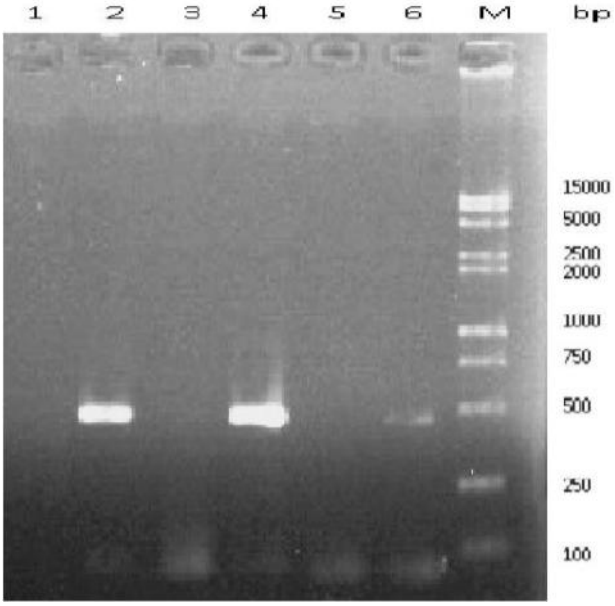

Figure 2. Analysis of PCR products by agarose gel electrophoresis for screening positive clone of pUC-fur

that the primers designed in this study could specifically bind to the total genomic DNA of strain A18 and the expected 500 bp fragments were amplified during PCR.

When the primers for amplification of the *fur* gene by PCR were designed, the start codon (ATG) and the stop codon (TAG) were inserted into the primers. Therefore, the amplified nucleotide sequence of the *fur* gene contained the whole Open Reading Frame (ORF). The analysis of the whole nucleotide sequence shows that ORF of the *fur* gene was composed of 447 bp. (Tsujibo *et al.* 2000). reported that the ORF of the *fur* gene from marine *Alteromonas* sp. O-7 has ATG start codon at 497.

The sequences of the *fur* genes from some negative bacteria available from GenBank and the sequence of the *fur* gene obtained in this study were aligned and the phylogenetic tree was constructed by using the software DNAstar. The phylogenetic tree shows that the nucleotide sequences reveal significant similarity with the *fur* genes from the same genus. For example, the similarity between the sequence of the *fur* gene from strain A18 used in this study and that from *Alteromonas* sp O-7 was 97%. This means that strain A18 was closely related to *Alteromonas* sp O-7 and the *fur* gene was highly conserved.

It can be observed from Figure 4 that the putative *fur* open reading frame coded for 148 amino acids representing a protein of 16.637 kDa. It has been reported the *fur* gene from *Alteromonas* sp.O-7 encodes a protein of 148 amino acids with a calculated molecular mass of 16.682 kDa (Tsujibo *et al.* 2000).The Fur protein of *E. coli* is a cytoplasmic 17 kDa polypeptide which binds iron as corepressor and consequently binds to the Fur-box, repressing gene transcription under iron-rich conditions (Escolar *et al.*, 1998). Then, the amino acid sequences of the Fur protein from some negative bacteria available from GenBank and the sequence of the Fur protein from strain A18 were aligned and the

GTAATACGACTCACTATAGGGCGACATATGATCGATGATATCCCATGGGCGGCCGCCTGC 60
AGACCAGGTCTTGAA ATG ACA GAC CAC AAC TTA GAG CTT AAA AAG GCT GGT TTA AAA GTA 120
ACG CTT CCA CGC ATC AAA ATT TTA GAG ATC CTT CAG TCT CCT GGT AAT CAA CAC ATC AGC 180
GCA GAA GAT GTG TAC AAG ATC TTA TTG GAC AAA GGT GAA GAA ATT GGC CTA GCT ACG GTA 240
TAT CGC GTA CTA AAC CAA TTT GAT GAT GCT GGT ATT GTA ACA CGT CAC CAC TTT GAA GGC 300
GGT AAA TCT GTT TTC GAG CTG TCT GGC AGC ACG CAC CAT GAT CAC TTG GTA TGT TTA AAA 360
TGT GGC AAA GTT GTC GAA TTT GAA GAT GAC GTT ATT GAA ACA CGC CAA GAA GAA ATC GCG 420
AAC AGC AAT GGC ATC AAG CTA ACA AAT CAC TCA CTA TAT CTA TAC GGT GAG TGC GAA GAT 480
AAA GAA GCG TGC AAA AAG TAC GCT GAG GAA AAT GGT AAC TAG CCTTCCACTTCGCCAAGA 540
CTGGAGATCTGGATCCCTCGAGTCTAGAGTCGACCTGCANGCATGCAAGC 590

Figure 3. Nucleotide sequence of the *fur* gene from *A. aurantia* A18

MTDHNLELKK AGLKVTLPRI KILEILQSPG NQHISAEDVY KILLDKGEEI GLATVYRVLN 60
QFDDAGIVTR HHFEGGKSVF ELSGSTHHDH LVCLKCGKVV EFEDDVIETR QEEIANSNGI 120
KLTNHSLYLY GECEDKEACK KYAEENGN 148

Figure 4. The deduced amino acid sequence of the Fur protein from A. aurantia

Table 2. The amount of siderophore in the cultures

Media	Strains	The amount of siderophore (%)
With 2,2-dipyridyl	QC1732	93.2±1.15
	QC1732(pQE30-fur)	92.5±1.15
	QC1732(pQE30-fur) induced by IPTG	92.1±0.40
Without 2,2-dipyridyl	QC1732	49.6±2.51
	QC1732(pQE30-fur)	45.4±0.81
	QC1732(pQE30-fur) induced by IPTG	30.9±1.37*

Values are means± SD; * Means differed ($P < 0.05$).

phylogenetic tree was constructed by using the software DNAstar. The results of the phylogenetic analysis (data not shown) show that the similarity between the amino acid sequence of Fur protein from *A. aurantia* A18 and that from *Alteromonas* sp O-7 was 98.6% whereas the similarity between the aminci acid sequence of Fur protein from *A. aurantia* A18 and that from *E. coli, S. oneidensis* MR-1, *Y. pestis* was 70.3%, 72.7% and 68.2%, respectively. The results of the phylogenetic analysis (data not shown) also show that the similarity between the amino acid sequence of Fur protein from *A. aurantia* A18 and that from three species of *Vibrio* was over 69%, whereas the similarity between the amino acid sequences of Fur proteins from different species of *Vibrio* was over 91%. This again proves that strain A18 was closely related to *Alteromonas* sp O-7 and the Fur proteins from different Gram negative bacteria were highly conserved.

E. coli QC1732 is a *fur* null mutant strain and thus, under iron-rich conditions, there is no repression of the Fur-regulated genes including those for siderophore biosynthesis and secretion. After the recombinant expression vector pQE30-fur constructed was transformed into the competent cells of *E. coli* QC1732, the *fur* gene in *E. coli* QC1732 (pQE30-fur) was confirmed by PCR (Figure 5).

When the single colony of *E. coli* QC1732 (pQE30-fur) was cultivated in liquid LB medium plus 50 $mg \cdot ml^{-1}$ of Ampicilin and Kanamycin at 37°C and OD_{600nm} value of the culture reached to 0.5, expression of the *fur* gene in *E. coli* QC1732 (pQE30-fur) was induced by adding $1mmol \cdot l^{-1}$ of IPTG for 10 h. Then the amount of siderophore in the supernatant of the culture was determined (Table 2). The culture from a single colony of *E. coli* QC1732 without pQE30-fur was used as the control (Table 2). In addition in order to reduce the amount of Fe^{2+} in the culture medium, 2,2-dipyridyl (the final concentration of it was 150 mm) was added to the medium to chelate iron Aso H et al. 2002),

The results in Table 2 indicate that when iron

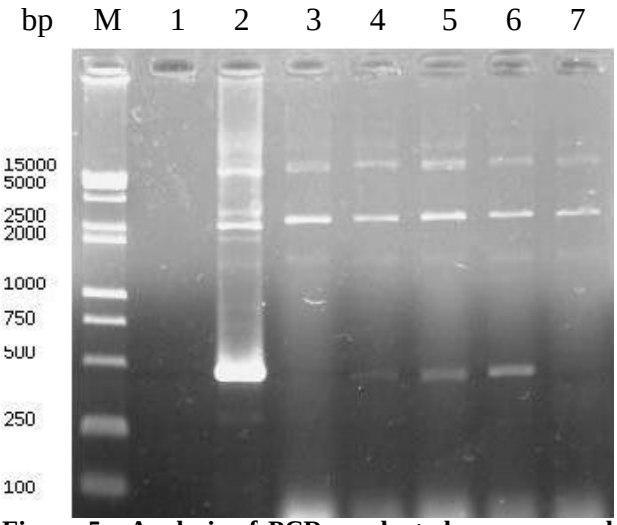

Figure 5. Analysis of PCR products by agarose gel electrophoresis for positive clone of pQE30-fur

was scare, the amount of siderophore in the 2,2-dipyridyl-adding cultures of *E. coli* QC1732, *E. coli* QC1732 (pQE30-fur) and QC1732 (pQE30-fur) induced by IPTG, was almost the same. This means that siderophore biosynthesis in *E. coli* was derepressed in the iron-deficient medium, resulting in increase in the amount of siderophores in the medium plus 2, 2-dipyridyl.However, the amount of siderophore was greatly reduced in the cultures grown in the iron-rich media, suggesting that siderophore biosynthesis in *E. coli* grown in the iron-rich medium was significantly repressed.

CONCLUSION

The amplified nucleotide sequence of the fur gene is contained 447bp. The homology analysis proved that the strain A18 is closely related to Alteromonas sp O-7. Statistically significant reduction of siderophore production by *E coli* strain with fur gene expression vector was observed when induced with IPTG.

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