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(54) Title: METHOD OF DETECTING, IDENTIFYING AND COUNTING ENTERITIS VIBRIO USING GENE (rpoD) SEQUENCES ENCODING RNA POLYMERASE σ70 FACTOR

(54) 発明の名称: RNAポリメレースσ70因子をコードする遺伝子（rpoD）配列を用いた腸炎ビブリオ検出、同定、計数方法

(57) Abstract: It is intended to construct a high-performance specific gene amplification primer for detecting, quantifying and identifying enteritis vibrio which has practically satisfactory amplification efficiency and amplification specificity and shows a low possibility of judgment error. The base sequences of RNA polymerase σ70 factor rpoD genes of a standard strain of *Vibrio* and enteritis vibrio stock cultures (a toxin gene-containing strain and a toxin gene-free strain) are determined and the systemic relationships among them are clarified. Thus, bases characteristic to enteritis vibrio are identified and a highly specific probes having these bases and a gene amplification primer having high specificity and high amplification efficiency can be designed.

(57) 要約:

誤判定の可能性が低く、かつ実用上十分な増幅効率と増幅特異性を有する、高性能な腸炎ビブリオの検出・定量・同定用特異遺伝子増幅プライマーの作製すること。

本発明者等は、ビブリオ属標準菌株及び腸炎ビブリオ保存株(毒素遺伝子保有株及び非保有株)のRNAポリメラーゼσ70因子rpoD遺伝子塩基配列を決め、その系統関係を明らかにし、腸炎ビブリオに特徴的塩基を同定し、これを含む高特異性を有するプローブ、及び高特異性を有しかつ増幅効率に優れた遺伝子増幅用プライマーを設計することを可能とした。

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Title of the Invention Method for Detecting, Identifying and Counting *Vibrio parahaemolyticus* using Gene (rpoD) Sequence Encoding RNA Polymerase σ70 Factor

Detailed Description of the Invention

Field of the Invention

The present invention relates to the field of identifying and testing microoraganisms and pathogenic microbes using genes, and particularly, testing *Vibrio parahaemolyticus*. The present invention relates to a method for rapidly identifying and testing *Vibrio parahaemolyticus* that causes food poisoning in the summer season, so that the field of the present invention includes food processing, public health and clinical laboratory testing.

Background Art

At the site of food hygiene control, *Vibrio parahaemolyticus* is currently tested based on biochemical phenotypes. According to the inspection guidelines for food hygiene, test samples are inoculated and cultured on TCBS agar media so as to obtain the thus formed greenish blue putatively positive colonies which are unable to degrade saccharose, and then various tests to identify the biochemical properties are conducted on the greenish blue colonies for determination. However, there are many problems in conducting the tests to identify the biochemical properties at the site of food manufacturing control, because the tests require accomplished techniques and a great deal of time and labor. In order to correct the defects of the standard tests to identify the biochemical properties, development of a test method using genes has been attempted for the purpose of detecting and identifying *Vibrio parahaemolyticus* accurately, rapidly and simply.

Disclosure of Invention

The following test methods using genes for testing *Vibrio parahaemolyticus* have been developed so far, but they involve various problems.

For example, PCR primers have been developed for detecting *Vibrio*

parahaemolyticus using as a target gyrB gene which encodes DNA gyrase β subunit (Japanese Patent Application Laying-Open (Kokai) No. 09-252783, Applied and Environmental Microbiology, Vol.64, No.2, P.681-687). However, the primers are prepared by assuming sections of sequences which are different between *Vibrio parahaemolyticus* strain ATCC 17802 and *Vibrio alginolyticus* strain ATCC17749 as specific, as a result of sequence analysis conducted on the gyrB gene of each single strain. In other words, the primers have not been prepared based on the understanding of the phylogenetic relation of the genus Vibrio based on the gyrB gene sequence, so that the range of specificity is unclear. Also reported is a detection method which uses a PCR method using as a target toxR gene that has been found as a gene controlling a toxin gene of *Vibrio cholera* and known to be present also in *Vibrio parahaemolyticus* (Journal of Clinical Microbiology. 1999 Vol. 37 No. 4, p1173-1177). However, similar to the above detection primers using gyrB gene, PCR primers of this example are prepared by comparing the gene sequences of each strain of *Vibrio parahaemolyticus* and *Vibrio cholera*, which are relatively distant from each other among phyla, and then assuming as *Vibrio parahaemolyticus*-specific sequences, sections of the nucleotide sequences that are different between the two strains. Hence, these primers have also not been prepared based on the understanding of the phylogenetic relation based on the toxR gene sequence, so that the range of specificity is unclear. On the other hand, there is another detection method noticing a toxin gene of *Vibrio parahaemolyticus* (Japanese Patent Application Laying Open (Kokai) No. 4-293486). It is long known that *Vibrio parahaemolyticus* is divided into a type having a toxin (Thermostable direct haemolysin: TDH) which causes hemolysis by boring holes on the membranes of red blood cells (referred to as Kanagawa phenomenon), and a type having no such toxin. In addition to TDH, a recently found toxin is TRH (TDH related haemolysin: TRH) which is very similar to TDH and causes no Kanagawa phenomenon but causes diarrhea (1988: Infect Immun. Vol. 56, 961-965). PCR primers have been developed for specifically detecting tdh and trh genes encoding each toxin that is thought to cause the pathogenicity of *Vibrio parahaemolyticus* (Japanese Patent Application Laying Open (Kokai) No. 4-293486). However, not all constituents of *Vibrio parahaemolyticus* possess such a toxin gene. Most constituents of *Vibrio parahaemolyticus* derived from environment actually possess no such toxin gene. Since the total cell count of all

constituents of *Vibrio parahaemolyticus* including strains that possess no toxin gene is considered important at the site of food hygiene control, the inspection guidelines for food hygiene require testing *Vibrio parahaemolyticus* regardless of the presence or absence of toxin. Accordingly, detection methods for a toxin gene, which notice toxin-production ability only, have no compatibility with standard methods which are based on biochemical examination, so that such detection methods are inappropriate as a detection and identification method at the site of food hygiene control. As described above, the practical use of currently available primers for detecting and identifying *Vibrio parahaemolyticus* is insufficient.

In addition to these detection methods, there have been reported methods for detecting a 0.76 Kb DNA fragment (Appl. Environ. Microbiol. 61(4):1311-1317) with unknown functions that is specifically present in *Vibrio parahaemolyticus* or hemolytic factors, such as tlh (Thermolabile haemolysin: Lett Appl Microbiol 1999, Vol.28, 66-70) and σ-VPH (FEMS Microbiol Lett 1990; 55(3):339-45). However, none of these methods have been proved to be able to reliably detect *Vibrio parahaemolyticus*, and none of them can be actually used at the site of evaluation.

As described above, none of the genetic testing methods considers that bacterial "species" is a population containing genetic diversity, and a nucleotide sequence of a strain assumed to be a member of a certain bacterial population is used as a common sequence or a representative sequence of the population for designing PCR primers. However, gene mutation, which is rapidly accumulated by molecular evolution, particularly neutral mutation, being taken into consideration, a strain originally to be detected cannot always be detected because amplification may be inhibited if slight mutation in primer region decreased applicability as a primer. Moreover, it is feared that there may be misidentitication, because the lack of consideration on differences with closely related species by phylogenetic analysis inhibits design of a primer having sufficient amplification specificity, such that an closely related strain that is not originally a target is detected.

Thus, the preparation of high-performance, specific gene-amplification primers

for detecting, identifying and quantitatively determining *Vibrio parahaemolyticus* having a proven background for specificity, low possibility of misidentitication, and practically sufficient amplification efficiency and specificity has been required.

To establish a method for specifically detecting a gene of a certain phylogenetic group of bacteria, it is required to collect and compare as many as possible nucleotide sequences of an organism group to be detected, and of an organism group which is phylogenetically close to the group. In addition, a gene targeted for specific detection is required to have a sufficiently different nucleotide sequence to enable discrimination from the closest relatives. Thus, a target gene must have a sufficiently rapid rate of evolution.

Further, a gene which is present independently from phylogeny, such as a toxin gene of *Vibrio parahaemolyticus*, that is spread horizontally at a high frequency, cannot be used. A σ70 factor encoded by rpoD gene that is used as a target in the present invention is a factor controlling gene expression in bacteria at the logarithmic phase, and is a protein essential for survival. Therefore the factor is appropriate for phylogenetic analysis of bacteria, because it is rarely spread horizontally and has an appropriate rate of evolution (Int. J. Syst. Bacteriol. 1998; 48, 813-819, Int. J. Syst. Bacteriol. 1999; 49, 87-95).

Summary of the Invention

In one aspect of the present invention, there is provided a fragment of a gene represented by SEQ ID NO: 1 that can be used for designing specific gene-amplification primers or probes, which comprises any nucleotides, unique to the groups of Vibrio parahaemolyticus, of position Nos. 33, 93, 102, 123, 141, 147, 148, 192, 204, 223, 229, 234, 243, 259, 261, 264, 267, 270, 384, 390, 501, 594, 597, 633, 712, 735, and 798 in the gene (rpoD) encoding RNA polymerase σ70 factor of SEQ ID NO: 1 in the Sequence Listing.

In another aspect of the present invention there is provided a gene amplification primer, which comprises a strand consisting of 15 or more consecutive nucleotides containing any nucleotides, unique to the groups of Vibrio parahaemolyticus, of position Nos. 33, 93, 102, 123, 141, 147, 148, 192, 198, 204, 223, 229, 234, 243, 259, 261, 264, 267, 270, 384, 390, 501, 594, 597, 633, 712, 735, and 798 in the gene (rpoD) encoding RNA polymerase σ70 factor of SEQ ID NO: 1 in the Sequence Listing, or a complementary strand corresponding thereto.

In another aspect of the present invention, there is provided a method for detecting, quantitatively determining or identifying Vibrio parahaemolyticus, which uses the primer described herein.

In yet another aspect of the present invention, there is provided a kit comprising the primer as described herein.

In yet another aspect of the present invention, there is provided a probe for detecting, quantitatively determining or identifying Vibrio parahaemolyticus, which comprises 15 or more consecutive nucleotides containing any nucleotides, unique to the groups of *Vibrio parahaemolyticus*, of position Nos. 33, 93, 102, 123, 141, 147, 148, 192, 198, 204, 223, 229, 234, 243, 259, 261, 264, 267, 270, 384, 390, 501, 594, 597, 633, 712, 735 and 798 in the gene (rpoD) encoding RNA polymerase σ70 factor of SEQ ID NO: 1 in the Sequence Listing.

Brief Description of Drawings

Fig. 1. Phylogenetic tree of the genus Vibrio based on rpoD gene sequence

Fig. 2 Phylogenetic analysis of food-derived, *Vibrio parahaemolyticus*-like colonies based on rpoD gene

Phylogenetic trees constructed by the neighbor - joining method (NJ) after analysis of an approximately 800 bp partial sequence of rpoD gene determined by direct sequencing method. Strains determined as *Vibrio parahaemolyticus* based on analysis of biochemical properties (the primary and secondary identification tests were

performed according to the inspection guidelines for food hygiene) were described as *V. parahaemolyticus*. The phyletic group that *Vibrio parahameolyticus* belongs to is denoted as V. p, and other phyletic proups of the genus Vibrio are classified into C1 to 5 as shown in the figure. The rpoD gene sequence of *E. coli* strain K12 and that of *V. cholerae* used herein were accession Nos. AE000388 and AE004138 of the GenBank database.

Fig. 3 Information on specificities among the phyla of the nucleotide sequences of rpoD gene of the genus Vibrio

The figure shows results after determination of the consensus sequences of the phyla V. p and C-1 to 3 (shown in Fig. 1), followed by homology analysis.
The upper case is V. p (the phylum that *Vibrio parahaemolyticus* belongs to); the lower case is the consensus sequence of the phyla C1 to 3. A section indicated by small sequential * denotes that it is the same sequence as the above sequence. A section indicated by large • denotes that it is a nucleotide specific to *Vibrio parahaemolyticus*. Symbols indicated are D=A or G or T; H=A or C or T; V=A or C or G; R=A or G; Y=C or T, K=G or T, M=A or C, S=G or C, W=A or T, and N=A or G or T or C.

Best Mode for Carrying Out the Invention

We have already developed a simple method for determining nucleotide sequences of rpoD gene which encodes RNA polymerase σ70 factor by a PCR direct sequencing method (Japanese Patent Application Laying Open (Kokai) No. 8-256798). (Table 1)

Therefore, using the method, we have determined the nucleotide sequences of rpoD gene of the type strains of the genus Vibrio (purchased from Institute for Fermentation, Osaka (IFO)) and *Vibrio parahaemolyticus* stock strains (strains containing and those not containing toxin gene) as shown in Table 1 above, thereby clarifying the phylogenetic relation. Specifically, test strains shown in Table 1 were cultured for growth on brain/heart infusion media (NISSUI PHARMACEUTICAL Co., Ltd) supplemented with 2% NaCl at 35°C overnight. Chromosome DNA was extracted from

1 ml of the culture solution using PUREGENE DNA Isolation Kit (Gentra SYSTEMS). Using the extracted DNAs as templates and rpoD amplification universal primers (s70S:ACgACTgACCCggTACgCATgTAYATgMgNgARATgggNACNgT and s70R:ATAgAAATAACCAgACgTAAgTTNgCYTCNACCATYTCYTTYTT described in Japanese Patent Application Laying Open (Kokai) No. 8-256798), an rpoD gene fragment of approximately 800 bp (positions 334-1125 on rpoD gene sequence of *Escherichia coli* strain K-12, corresponding to a region of positions 112 to 376 of an amino acid sequence) was amplified by a PCR method. Amplification reaction was performed using thermostable DNA polymerase (AmpliTaq Gold: Applied Biosystems) and a GENE MATE thermal cycler (ISC BioExpress). 50μl of a reaction solution was prepared to contain 1 μg of DNA, 50mM KCl, 10mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2mM of each dNTP, 2.5U AmpliTaq Gold and 1μM of each primer. The reaction condition consisting of activation (95°C for 10 min) with AmpliTaq Gold, 40 cycles of reaction (94°C for 1 min, 56°C for 1 min and 72°C for 1 min), and then elongation reaction (72°C for 10 min) was performed. The resulting PCR products were subjected to 1% agarose gel (Sea Plaque GTG agarose: BioWhittaker Molecular Applications) electrophoresis (0.5xTAE, 100V for 30 min), and then stained with ethidium bromide for 10 min. The presence of the product was confirmed under ultraviolet radiation, excised from the gel, and then purified using Wizard PCR Preps DNA Purification System (Promega), thereby preparing a template for sequence reaction. Sequence reaction was performed using sequences for sequence reaction (s70sS :ACgACTgACCCggTACgCATgTA and s70sR:ATAgAAATAACCAgACgTAAgTT described in Japanese Patent Application Laying Open (Kokai) No. 8-256798) previously added to the above universal primers for rpoD gene as primers, ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and GENE MATE thermal cycler (ISC BioExpress). The reaction solution was prepared to have a final volume of 20 μl by mixing 20 ng of DNA, 3.2 pmol of primers and 8 μl of BigDye Terminator Ready Reaction Mix. The cycle sequence reaction consisting of heating at 92°C for 10 min, and 25 cycles of 96°C for 10 sec, 58°C or 46°C (corresponding respectively to s70sS:ACgACTgACCCggTACgCATgTA and s70sR:ATAgAAATAACCAgACgTAAgTT) for 5 sec, and 60°C for 4 min was performed. For nucleotide sequence analysis, ABI PRISM 310 GENETIC ANALYZER (Applied

Biosystems) was used. Multiple alignment analysis was performed for the obtained nucleotide sequences using Clustal W computer program, and then phylogenetic trees were constructed by the neighbor - joining method (Mol. Biol. Evol. Vol.4, No. 4, 406-425) based on the genetic distance calculated with PHYLIP computer program package and Kimura's 2 - parameter model (J. Mol. Evol, (1980) Vol.16, No.2, p.111-20).

As a result, it was shown that all the strains determined as *Vibrio parahaemolyticus* containing toxin genes (tdh or trh) and a type strain (IFO 12711 ^{T}) together compose an independent mono phyletic group, in the strains of the genus Vibrio (see Fig. 1). Therefore, it was suggested that constituents of bacteria belonging to this phylum should be determined as *Vibrio parahaemolyticus*. Next, for 64 strains which had been isolated from food samples and form *Vibrio parahaemolyticus*-like greenish blue colonies on TCBS agar media (NISSUI PHARMACEUTICAL CO., LTD), the nucleotide sequences of rpoD gene were determined by procedures similar to those used for the type strains, and then phylogenetic analysis was conducted. Figure 2 shows the results. 11 out of 64 strains analyzed belonged to the phylum that should be determined as the above *Vibrio parahaemolyticus*. To verify the results of the gene analysis, biochemical properties were identified by performing examination according to the primary and secondary identification tests described in the guidelines for food inspection. Specifically, the primary identification test was performed for each of the parameters of oxidase, lactose/saccharose degradation ability using TSI media, gas generation ability upon glucose degradation, hydrogen sulfide production ability, indole production ability using SIM media, indole pyruvic acid production ability, confirmation of motility, lysine decarboxylase activity, Voges-Proskauer test and salt-tolerance (0, 3, 7 and 10% NaCl); the secondary identification test was performed for each of the parameters of ONPG, ornithine decarboxylase activity, arginine dehydrase activity and fermentation ability for various sugars (arabinose, maltose, inosit, xylose, salicin, mannite, mannose, lactose and saccharose). In the primary identification test, *Vibrio parahaemolyticus* was shown to have the following properties; oxidase, positive; lactose/saccharose degradation in TSI media, no degradation; hydrogen sulfide, no production; gas generation upon glucose degradation, none; indole production ability on SIM media, positive; indolepyruvic acid production ability, negative; motility, positive; lysine decarboxylase activity, positive; Voges-Proskauer test, negative; and

salt-tolerance (0, 3, 7 and 10% NaCl): −, + , + and −. In the secondary confirmation test, *Vibrio parahaemolyticus* was shown to have the following properties: ONPG, negative; ornithine decarboxylase activity, positive; arginine dehydrase activity, negative; sugar fermentation ability (arabinose, positive; maltose, positive; inosit, negative; xylose, negative; salicin, negative; mannite, positive; mannose, positive; lactose, negative and saccharose, negative). The above biochemical examination revealed that only 11 strains which had been derived from food and belonged to the phylum that should be determined as *Vibrio parahaemolyticus* according to the previous analysis of rpoD gene were determined as *Vibrio parahaemolyticus*. Therefore, it was shown that *Vibrio parahaemolyticus* groups can be distinguished and identified accurately by analysis of rpoD gene.

To establish a genetic screening method capable of detecting only *Vibrio parahaemolyticus* constituents, the following procedures were performed. First, to clarify differences in nucleotide sequences among the closely related species, the nucleotide sequences of rpoD gene of the phylum that *Vibrio parahaemolyticus* belongs to and the neighbor phyla were compared, and then positions of nucleotides which are conserved among *Vibrio parahaemolyticus* constituents but differ from those of other bacteria belonging to the genus Vibrio were identified. Specifically, a consensus sequence of phyletic group that *Vibrio parahaemolyticus* belongs to was determined, while the consensus sequence was compared with those of the cluster C-1 to C-3, which are other pyletic groups of bacteria belonging to the genus Vibrio as shown by Fig. 2 to be phylogenetically close to the phylum that Vibrio parahaemolyticus belongs to. Thus, phylogenetically specific information was constructed (Fig. 3). It was shown that nucleotide positions 33, 93, 102, 123, 141, 147, 148, 192, 198, 204, 223, 229, 234, 243, 259, 261, 264, 267, 270, 384, 390, 501, 594, 597, 633, 712, 735 or 798 of SEQ ID NO: 1 in the Sequence Listing are characteristic in the phylum Vibrio parahaemolyticus belongs to. The use of a specific sequence of the this phylum containing these characteristic nucleotides enables the design of probes having high specificity and primers for gene amplification which have high specificity and excellent amplification efficiency.

For example, primers can be designed to always contain a position(s) at which a nucleotide(s) is different from that of closely related species, using the nucleotide sequence of rpoD gene comprising 15 or more consecutive nucleotides containing a nucleotide(s) different from that of the closely related species, preferably, 20 nucleotides or more, and further preferably, 20 nucleotides or more and 40 nucleotides or less. Similarly, probes can be designed to always contain a position(s) at which a nucleotide(s) is different from that of closely related species, using the nucleotide sequence of rpoD gene comprising15 or more consecutive nucleotides containing a nucleotide(s) different from that of the closely related species, preferably, 20 nucleotides or more, and further preferably, 20 nucleotides or more and 100 nucleotides or less.

Further, regions that can be preferably used to prepare the primers and probes contain at a high frequency the above nucleotide(s) different from that of closely related species, for example, a region containing 259, 261, 264, 267 and 270; a region containing 141, 147 and 148; a region containing 192, 198 and 204: a region containing 223, 229 and 234; and a region containing 594 and 597. For primers, the 3' terminus is preferably a nucleotide specific to *Vibrio parahaemolyticus*.

The gene amplification primers of present inventions may be used for detecting, quantitatively determining or identifying Vibrio parahaemolyticus. The present invention encompasses a kit for detecting, quantitatively determining or identifying *Vibrio parahaemolyticus* which comprises these primers and probes and other reagents in combination.

Table 1. List of Strains Used

Type strains of genus Vibrio	8 strains	*V. parahaemolyticus*	IFO 12711 T
		V. alginolyticus	IFO 15630 T
		V. proteolyticus	IFO 13287 T
		V. nereis	IFO 15637 T
		V. campbellii	IFO 15631 T
		V. harveyi	IFO 15634 T
		V. carchariae	IFO 15632 T
		V. tubiashii	IFO 15644 T
Stock Strains of Vibrio parahaemolyticus	Derived from Human 13 strains	V89-655 O3:K6	TRH:+
		V89-056 O4:K8	TDH:+
		V99-157 O1:K56	TDH:+
		V99-161 O4:K11	TDH:+
		V99-177 O4:K8	TDH:+
		V99-215 O3:K6	TDH:+
		V99-223 O4:K9	TDH:+
	Derived from Food	No toxin production: 6 strains	
Isolated strains on TCBS media	64strains	Derived from food	
Total	**85 strains**		

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Examples

The present invention will now be further described by means of examples. However, the examples represent embodiments of the present invention, and are not intended to limit the invention.

Specific detection of *Vibrio parahaemolyticus* was attempted by a PCR method using primers for detecting and identifying *Vibrio parahaemolyticus* shown in Table 2. In addition, primers described in Claims 10 to 15 are F1, F2, F5, F6, R1 and R2, respectively. Chromosome DNAs extracted from the test strains shown in Table 1 above were used as templates. Table 2 shows details about primers used. Amplification reaction was performed using thermostable DNA polymerase (AmpliTaq Gold : Applied Biosystems) and a GENE MATE thermal cycler (ISC BioExpress). A reaction solution was prepared to have a final volume of 20 μl and contain DNA 0.1 μg, 50mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin, dNTP (0.2 mM each), 2.5 U of AmpliTaq Gold and primers (see Table 3 for concentration). A reaction condition consisted of activation with AmpliTaq Gold (95°C for 10 min); 35 to 40 reaction cycles of 94°C for 1 min, annealing for 1 min (see Table 3 for temperature), and 72°C for 1 min; and then elongation reaction at 72°C for 10 min was performed. Table 3 shows primer combinations and amplification reaction conditions. 5 μl of the reaction solution after amplification was subjected to 2% agarose gel (agarose S: NIPPON GENE CO., LTD.) electrophoresis (0.5xTAE, 100V for 30 min), and then stained with ethidium bromide for 10 min, followed by confirmation of the presence or absence of amplified rpoD genes under ultraviolet radiation.

Using 4 types of sense primers and 2 types of antisense primers, that is, 8 combinations of primers in total, DNAs of the test strains were screened. Thus, only the DNAs belonging to the phylum that should be determined as *Vibrio parahaemolyticus* were positive (Table 4).

Table2. Primers for specific detection of Vibrio parahaemolyticus

Primer	Sequence	Length	Position[a]	Direction
F1	agarcttcgtctgactgatt	20	129 - 148	Sense
F2	aagaagacctagaagatgat	20	251 - 270	Sense
F5	cagcwgcgccaaccgcgact	20	185 - 204	Sense
F6	ctgarctgtctgaarctcaa	20	215 - 234	Sense
R1	gttaccagtgaatagggca	19	609 - 591	Antisense
R2	attcgttaccagtgaatagg	20	613 - 594	Antisense

a 。indicates a position from the 5' terminus in the nucleotide sequence represented by SEQ

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Table 3. PCR conditions for primers for specific detection of Vibrio parahaemolyticus

Sense primer	Antisense primer	Amplified product (bp)	PCR conditions		
			Annealing temperature (°C)	Number of cycles	Primer concentration (mM)
F1	R1	483	58	35	0.1
F2	R1	361	56	40	0.1
F5	R1	427	64	40	0.1
F6	R1	397	60	35	0.1
F1	R2	485	58	40	0.1
F2	R2	365	60	40	0.5
F5	R2	431	60	40	0.1
F6	R2	401	56	40	0.1

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Table 4 PCR result using primers for specific detection of Vibrio parahaemolyticus

Sequence number	Cluster	Name	Strain name	Kanagawa phenomenon	PCR							
					F1 & R1	F2 & R1	F5 & R1	F6 & R1	F1 & R2	F2 & R2	F5 & R2	F6 & R2
1	V.p	*V. parahaemolyticus*	V99-161	+	+	+	+	+	+	+	+	+
2	V.p	*V. parahaemolyticus*	IFO 12711 T	−	+	+	+	+	+	+	+	+
3	V.p	*V. parahaemolyticus*	V5	−	+	+	+	+	+	+	+	+
4	V.p	*V. parahaemolyticus*	V99-10	−	+	+	+	+	+	+	+	+
5	V.p	*V. parahaemolyticus*	V73	−	+	+	+	+	+	+	+	+
6	V.p	*V. parahaemolyticus*	V99-55	−	+	+	+	+	+	+	+	+
7	V.p	*V. parahaemolyticus*	V99-78	−	+	+	+	+	+	+	+	+
8	V.p	*V. parahaemolyticus*	V20	−	+	+	+	+	+	+	+	+
9	V.p	*V. parahaemolyticus*	V17	−	+	+	+	+	+	+	+	+
10	V.p	*V. parahaemolyticus*	V47	−	+	+	+	+	+	+	+	+
11	V.p	*V. parahaemolyticus*	V99-223	+	+	+	+	+	+	+	+	+
12	V.p	*V. parahaemolyticus*	V89-655	−	+	+	+	+	+	+	+	+
13	V.p	*V. parahaemolyticus*	V48	−	+	+	+	+	+	+	+	+
14	V.p	*V. parahaemolyticus*	V99-215	+	+	+	+	+	+	+	+	+
15	V.p	*V. parahaemolyticus*	V46	−	+	+	+	+	+	+	+	+
16	V.p	*V. parahaemolyticus*	V72	−	+	+	+	+	+	+	+	+
17	V.p	*V. parahaemolyticus*	V83	−	+	+	+	+	+	+	+	+
18	V.p	*V. parahaemolyticus*	V81	−	+	+	+	+	+	+	+	+
19	V.p	*V. parahaemolyticus*	V78	−	+	+	+	+	+	+	+	+
20	V.p	*V. parahaemolyticus*	V84	−	+	+	+	+	+	+	+	+
21	V.p	*V. parahaemolyticus*	V69	−	+	+	+	+	+	+	+	+
22	V.p	*V. parahaemolyticus*	V99-157	+	+	+	+	+	+	+	+	+
23	V.p	*V. parahaemolyticus*	V89-056	+	+	+	+	+	+	+	+	+
24	V.p	*V. parahaemolyticus*	V99-15		+	+	+	+	+	+	+	+
25	V.p	*V. parahaemolyticus*	V99-177	+	+	+	+	+	+	+	+	+
26	C-2	*V. harveyi*	IFO 15634 T	−	−	−	−	−	−	−	−	−
27	C-2	*Vibrio* spp.	V33	−	−	−	−	−	−	−	−	−
28	C-2	*V. carchariae*	IFO 15632 T	−	−	−	−	−	−	−	−	−
29	C-2	*Vibrio* spp.	V80	−	−	−	−	−	−	−	−	−
30	C-2	*Vibrio* spp.	V74	−	−	−	−	−	−	−	−	−
31	C-2	*Vibrio* spp.	V15	−	−	−	−	−	−	−	−	−
32	C-2	*Vibrio* spp.	V3	−	−	−	−	−	−	−	−	−
33	C-2	*Vibrio* spp.	V42	−	−	−	−	−	−	−	−	−
34	C-2	*Vibrio* spp.	V44	−	−	−	−	−	−	−	−	−
35	C-2	*Vibrio* spp.	V41	−	−	−	−	−	−	−	−	−
36	C-2	*Vibrio* spp.	V89	−	−	−	−	−	−	−	−	−
37	C-2	*Vibrio* spp.	V87	−	−	−	−	−	−	−	−	−
38	C-2	*Vibrio* spp.	V76	−	−	−	−	−	−	−	−	−
39	C-2	*Vibrio* spp.	V88	−	−	−	−	−	−	−	−	−
40	C-2	*Vibrio* spp.	V4	−	−	−	−	−	−	−	−	−

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Table 4 PCR result using primers for specific detection of Vibrio parahaemolyticus

Sequence number	Cluster	Name	Strain name	Kanagawa phenomenon	PCR							
					F1 & R1	F2 & R1	F5 & R1	F6 & R1	F1 & R2	F2 & R2	F5 & R2	F6 & R2
41	C-2	*Vibrio* spp.	V86	−	−	−	−	−	−	−	−	−
42	C-2	*V. campbellii*	IFO 15631 T	−	−	−	−	−	−	−	−	−
43	C-2	*Vibrio* spp.	V18	−	−	−	−	−	−	−	−	−
44	C-1	*Vibrio* spp.	V68	−	−	−	−	−	−	−	−	−
45	C-1	*Vibrio* spp.	V85	−	−	−	−	−	−	−	−	−
46	C-1	*Vibrio* spp.	V16	−	−	−	−	−	−	−	−	−
47	C-1	*V. alginolyticus*	IFO 15630 T	−	−	−	−	−	−	−	−	−
48	C-3	*Vibrio* spp.	V70	−	−	−	−	−	−	−	−	−
49	C-3	*Vibrio* spp.	V71	−	−	−	−	−	−	−	−	−
50	C-5	*Vibrio* spp.	V77	−	−	−	−	−	−	−	−	−
51	C-5	*Vibrio* spp.	V82	−	−	−	−	−	−	−	−	−
52	C-4	*Vibrio* spp.	V45	−	−	−	−	−	−	−	−	−
53	C-4	*Vibrio* spp.	V8	−	−	−	−	−	−	−	−	−
54	C-4	*Vibrio* spp.	V10	−	−	−	−	−	−	−	−	−
55	C-4	*Vibrio* spp.	V13	−	−	−	−	−	−	−	−	−
56	C-4	*Vibrio* spp.	V6	−	−	−	−	−	−	−	−	−
57	C-4	*Vibrio* spp.	V9	−	−	−	−	−	−	−	−	−
58	C-4	*Vibrio* spp.	V7	−	−	−	−	−	−	−	−	−
59	C-4	*Vibrio* spp.	V14	−	−	−	−	−	−	−	−	−
60	C-4	*Vibrio* spp.	V11	−	−	−	−	−	−	−	−	−
61	C-4	*Vibrio* spp.	V12	−	−	−	−	−	−	−	−	−
62	C-4	*Vibrio* spp.	V79	−	−	−	−	−	−	−	−	−
63	C-4	*Vibrio* spp.	V43	−	−	−	−	−	−	−	−	−
64	C-4	*V. tubiashii*	IFO 15644 T	−	−	−	−	−	−	−	−	−
65	C-4	*V. nereis*	IFO 15637 T	−	−	−	−	−	−	−	−	−
66	C-4	*Vibrio* spp.	V64	−	−	−	−	−	−	−	−	−
67	C-4	*Vibrio* spp.	V56	−	−	−	−	−	−	−	−	−
68	C-4	*Vibrio* spp.	V55	−	−	−	−	−	−	−	−	−
69	C-4	*Vibrio* spp.	V57	−	−	−	−	−	−	−	−	−
70	C-4	*Vibrio* spp.	V63	−	−	−	−	−	−	−	−	−
71	C-4	*Vibrio* spp.	V61	−	−	−	−	−	−	−	−	−
72	C-4	*Vibrio* spp.	V65	−	−	−	−	−	−	−	−	−
73	C-4	*Vibrio* spp.	V60	−	−	−	−	−	−	−	−	−
74	C-4	*Vibrio* spp.	V67	−	−	−	−	−	−	−	−	−
75	C-4	*Vibrio* spp.	V66	−	−	−	−	−	−	−	−	−
76	C-4	*Vibrio* spp.	V59	−	−	−	−	−	−	−	−	−
77	C-4	*Vibrio* spp.	V58	−	−	−	−	−	−	−	−	−
78	C-4	*Vibrio* spp.	V62	−	−	−	−	−	−	−	−	−
79	C-4	*Vibrio* spp.	V19	−	−	−	−	−	−	−	−	−
80	C-4	V. proteolyticus	IFO 13287 T	−	−	−	−	−	−	−	−	−

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Table 4 PCR result using primers for specific detection of Vibrio parahaemolyticus

Sequence number	Cluster	Name	Strain name	Kanagawa phenomenon	PCR							
					F1 & R1	F2 & R1	F5 & R1	F6 & R1	F1 & R2	F2 & R2	F5 & R2	F6 & R2
81		*Shewanella* spp.	V51	—	—	—	—	—	—	—	—	—
82		*Shewanella* spp.	V49	—	—	—	—	—	—	—	—	—
83		*Shewanella* spp.	V53	—	—	—	—	—	—	—	—	—
84		*Shewanella* spp.	V50	—	—	—	—	—	—	—	—	—
85		*Shewanella* spp.	V52	—	—	—	—	—	—	—	—	—

Industrial Applicability

The rpoD gene primers and probes of the present invention are excellent in terms of detection accuracy because they have been designed based on a thorough understanding of the phylogenetic relation with *Vibrio parahaemolyticus*, by which improvement of the specificity has been studied. Therefore, the rpoD gene primers and probes are of great advantage for direct detection under conditions where bacteria are not isolated from food, and allied bacterial species exist together.

Free text in Sequence listing

SEQ ID No.s 9-12 are primers.

Present specification incorporates by reference contents of specification including claims and drawings of patent application number 2001-235806 filed in the Japan Patent Office on August 3, 2001 on which priority is claimed.

Claims

Claim 1 A fragment of a gene represented by SEQ ID NO: 1 that can be used for designing specific gene-amplification primers or probes, which comprises any nucleotides, unique to the groups of *Vibrio parahaemolyticus*, of position Nos. 33, 93, 102, 123, 141, 147, 148, 192, 198, 204, 223, 229, 234, 243, 259, 261, 264, 267, 270, 384, 390, 501, 594, 597, 633, 712, 735 and 798 in the gene (rpoD) encoding RNA polymerase σ70 factor of SEQ ID NO: 1 in the Sequence Listing.

Claim 2 A gene amplification primer, which comprises a strand consisting of 15 or more consecutive nucleotides containing any nucleotides, unique to the groups of *Vibrio parahaemolyticus*, of position Nos. 33, 93, 102, 123, 141, 147, 148, 192, 198, 204, 223, 229, 234, 243, 259, 261, 264, 267, 270, 384, 390, 501, 594, 597, 633, 712, 735 and 798 in the gene (rpoD) encoding RNA polymerase σ70 factor of SEQ ID NO: 1 in the Sequence Listing, or a complementary strand corresponding thereto.

Claim 3 The gene amplification primer of claim 2, which uses a region containing at a high frequency positions unique to *Vibrio parahaemolyticus* as specified in Claim 2.

Claim 4 The gene amplification primer of claim 3, which comprises a strand containing nucleotides of position Nos. 259, 261, 264, 267 and 270 of SEQ ID NO: 1 in the Sequence Listing, or a complementary strand thereto.

Claim 5 The gene amplification primer of claim 3, which comprises a strand containing nucleotides of position Nos. 141, 147 and 148 of SEQ ID NO: 1 in the Sequence Listing, or a complementary strand thereto.

Claim 6 The gene amplification primer of claim 3, which comprises a strand containing nucleotides of position Nos. 192, 198 and 204 of SEQ ID NO: 1 in the Sequence Listing, or a complementary strand thereto.

Claim 7 The gene amplification primer of claim 3, which comprises a strand containing nucleotides of position Nos. 223, 229 and 234 of SEQ ID NO: 1 in the Sequence Listing, or a complementary strand thereto.

Claim 8 The gene amplification primer of claim 3, which comprises a strand containing nucleotides of position Nos. 594 and 597 of SEQ ID NO: 1 in the Sequence Listing, or a complementary strand thereto.

Claim 9 The gene amplification primer of claim 2, wherein a nucleotide at the 3' terminus is a *Vibrio parahaemolyticus*-unique nucleotide having a position number

specified in Claim 2.

Claim 10 The gene amplification primer of claim 2, which comprises 5'-agarcttcgtctgactgatt-3', or a complementary strand corresponding thereto.

Claim 11 The gene amplification primer of claim 2, which comprises 5'-aagaagacctagaagatgat-3', or a complementary strand corresponding thereto.

Claim 12 The gene amplification primer of claim 2, which comprises 5'-cagcwgcgccaaccgcgact-3', or a complementary strand corresponding thereto.

Claim 13 The gene amplification primer of claim 2, which comprises 5'-ctgarctgtctgaarctcaa-3', or a complementary strand corresponding thereto.

Claim 14 The gene amplification primer of claim 2, which comprises 5'-gttaccagtgaatagggca-3', or a complementary strand corresponding thereto.

Claim 15 The gene amplification primer of claim 2, which comprises 5'-attcgttaccagtgaatagg-3', or a complementary strand corresponding thereto.

Claim 16 A method for detecting, quantitatively determining or identifying *Vibrio parahaemolyticus*, which uses the primer of any one of Claims 2 to 15.

Claim 17 A kit comprising the primer of any one of Claims 2 to 15.

Claim 18 A probe for detecting, quantitatively determining or identifying *Vibrio parahaemolyticus*, which comprises 15 or more consecutive nucleotides containing any nucleotides, unique to the groups of *Vibrio parahaemolyticus*, of position Nos. 33, 93, 102, 123, 141, 147, 148, 192, 198, 204, 223, 229, 234, 243, 259, 261, 264, 267, 270, 384, 390, 501, 594, 597, 633, 712, 735 and 798 in the gene (rpoD) encoding RNA polymerase σ70 factor of SEQ ID NO: 1 in the Sequence Listing.

Abstract

To prepare high-performance specific gene-amplification primers for detecting, quantitatively determining or identifying *Vibrio parahaemolyticus* having low possibility of mis-identification and practically sufficient amplification efficiency and amplification specificity.

We have determined the nucleotide sequences of rpoD genes encoding RNA polymerase σ70 factor of type strains of the genus Vibrio and of stock strains of *Vibrio parahaemolyticus* (strains containing and those not containing toxin gene); elucidated the phylogenetic relation, and identified nucleotides which are characteristic in *Vibrio parahaemolyticus*, thereby enabling the design of probes containing them and having high specificity, and primers for gene amplification having high specificity and excellent amplification efficiency.

SEQUENCE LISTING

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<130> PH-1546-PCT

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<170> PatentIn Ver. 2.1

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caggcagaag arcttcgtct gactgattta atctctggct ttgtagatcc tgacgctgac 180

gatacagcwg cgccaaccgc gactcacatc ggttctgarc tgtctgaarc tcaattagaa 240

gaggaagacg aagaagacct agaagatgat gaagaragcg atgacgattc agatgaytcr 300

gaagaagatg taggtattga yccagagctr gcgcttgaga aattcaacca gctacgcagc 360

acataccaaa atcttcagct agcgatcaat gagtacggct acgacagccc gaaagcaacc 420

gttgctaacg aaatgatgct rgacgtattc aaagaattcc gtctaacacc aaaacagttc 480
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gctgagaaaa tcaaacgtaa cgaagaagag atccgtcgtt caatckckaa gttaaaaatg 720
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atcggtgaag cgaaagcacg ccgtgcg 807

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<220>
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caagcwgaag aaytdcgtct wacwgacctw atytcwgght ttgthgaycc wgaygchgay 180
gayacvrcwg chccracrgc racrcacaty ggttctgarc tractgaatc tcagytagaa 240
gawgaagayg awgaagacgt tgatgamgac gaagawrgyg aygayrrykm wgatgahdcw 300
gargaagatg twggtatyga yccwgarytd gcgctwgaga aattcaacca rctacgcagy 360
acvtaycaaa aycttcaryt wgcaatcaac garyacggyt ayravagycc kaaagcracm 420
gtwgcwaayg arwtgatgct agacgtatty mrmgarttyc gtctracrcc waaacagtty 480
gaycacytag traaygaayt rcgyacnkcd atggatcgyg ttcgtacwca agarcgyytg 540
atcatgaark cwryngttga atacggcaaa atgccgaaga aatcrttyat ygcrctgtty 600

acwggyaayg aatcwashga wgcwtggytr gatgarrtyy twkcwtcwga yaagccatac 660

gctgaraara tyaaacgtar cgaagaagar atycgycgyt caatcrctaa rctaaaratg 720

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