Sequence Analysis of Amplified DNA Fragments Containing the Region Encoding the Putative Lipase Substrate-Binding Domain and Genotyping of *Aeromonas hydrophila*

Noboru Watanabe,* Koji Morita, Tomoko Furukawa, Taki Manzoku, Eiko Endo, and Masato Kanamori

Department of Microbiology, Kyorin University School of Health Sciences, Tokyo 192-8508, Japan

Received 10 July 2003/Accepted 21 October 2003

DNA fragments were amplified by PCR from all tested strains of *Aeromonas hydrophila*, *A. caviae*, and *A. sobria* with primers designed based on sequence alignment of all lipase, phospholipase C, and phospholipase A1 genes and the cytotoxic enterotoxin gene, all of which have been reported to have the consensus region of the putative lipase substrate-binding domain. All strains showed lipase activity, and all amplified DNA fragments contained a nucleotide sequence corresponding to the substrate-binding domain. Thirty-five distinct nucleotide sequence patterns and 15 distinct deduced amino acid sequence patterns were found in the amplified DNA fragments from 59 *A. hydrophila* strains. The deduced amino acid sequences of the amplified DNA fragments from *A. caviae* and *A. sobria* strains had distinctive amino acids, suggesting a species-specific sequence in each organism. Furthermore, the amino acid sequence patterns appear to differ between clinical and environmental isolates among *A. hydrophila* strains. Some strains whose nucleotide sequences were identical to one another in the amplified region showed an identical DNA fingerprinting pattern by repetitive extragenic palindromic sequence-PCR genotyping. These results suggest that *A. hydrophila*, and also *A. caviae* and *A. sobria* strains, have a gene encoding a protein with lipase activity. Homologs of the gene appear to be widely distributed in *Aeromonas* strains, probably associating with the evolutionary genetic difference between clinical and environmental isolates of *A. hydrophila*. Additionally, the distinctive nucleotide sequences of the genes could be attributed to the genotype of each strain, suggesting that their analysis may be helpful in elucidating the genetic heterogeneity of *Aeromonas*.

Aeromonas hydrophila is widely distributed in the aquatic environment (1, 14) and has been implicated in human infectious diseases (20), such as gastroenteritis (11) and septicemia (21). *A. hydrophila* produces a variety of extracellular enzymes, including protease (22), elastase (6), amylase (15), chitinase (24), lipases (2, 9), phospholipases (18, 23), hemolysins (3, 17), and enterotoxins (7, 8, 26, 27).

Two lipase (*lip* and *lipH3*) genes, phospholipase C (*apl-1*) gene, phospholipase A1 (*pla*) gene, and cytotoxic enterotoxin (*alt*) gene were found from distinct strains of *A. hydrophila*. The *lip* and *lipH3* genes code for extracellular lipases, which are similar in that they have esterase but not phospholipase activities (2, 9). The *apl-1* gene codes for a lipase and exhibits nonhemolytic phospholipase C activity (18). The *pla* gene codes for a putative lipoprotein; it is thought that this lipoprotein is a lipase with phospholipase A1 activity but is nonhemolytic, noncytotoxic, and nonenterotoxic (23). Although physical properties of these enzymes, such as molecular size and thermal stability as well as the substrate specificities, differ from one another, the *lip*, *lipH3*, *apl-1*, and *pla* genes show similarities in their amino acid sequences (23). While the DNA size of the *alt* gene is about half that of the above genes, this gene shows amino acid similarity to the 3' half of the above genes (8, 23). The *alt* gene codes for a heat-labile cytotoxic enterotoxin;

the purified native toxin exhibits the ability to elongate CHO cells and to elicit fluid secretion in rat ligated intestinal loops but had no lipase or phospholipase C activity (8). These lipases, phospholipases, and cytotoxic enterotoxin have a consensus amino acid sequence, V-H-F-L-G-H-S-L-G-A, corresponding to the region of putative substrate-binding domains found within bacterial, fungal, porcine, and human lipases (12).

In this study we attempted to define the distribution of the genes which have the region corresponding to the putative lipase substrate-binding domain found in the *lip*, *lipH3*, *apl-1*, *pla*, and *alt* genes among clinical and environmental isolates of *A. hydrophila* belonging to serogroups O11, O16, and O34, which are considered to be the major O serogroups of the genus *Aeromonas* (19). PCR was applied to amplify the DNA fragments, including the region corresponding to that domain. The PCR products were then sequenced to identify the amplified DNA genes.

Genetic heterogeneity in *A. hydrophila* has been studied by methods such as random amplified polymorphic-PCR (10, 25) and enterobacterial repetitive intergenic consensus-PCR (10). Also, repetitive extragenic palindromic sequence-PCR (REP-PCR) is one of the useful genotyping methods to examine genetic heterogeneity (4). We compared the REP-PCR DNA fingerprints among the tested *A. hydrophila* strains and examined the correlation between the genetic heterogeneity and the nucleotide sequences of the above PCR products. In addition, we applied the above experimental analyses on strains of *A. caviae* and *A. sobria* to compare with *A. hydrophila* results.

* Corresponding author. Mailing address: Department of Microbiology, Kyorin University School of Health Sciences, 476 Miyashita-cho, Hachioji-shi, Tokyo 192-8508, Japan. Phone: 0426-91-0011. Fax: 0426-91-1094. E-mail: wtnbnbr@kyorin-u.ac.jp.

MATERIALS AND METHODS

Bacterial strains and genes. *Aeromonas* strains used in this study are described in Fig. 1. The profiles of the reference genes, *lip*, *lipH3*, *apl-1*, *pla*, and *alt*, are listed in Table 1.

Detection of lipase activity. Lipase activity of the tested strains was detected on tributyrin agar medium containing 0.5% peptone (Difco), 0.3% yeast extract (Difco), and 1.5% agar (Oxoid) supplemented with 1% tributyrin (18). After inoculation the tributyrin agar medium was incubated at 37°C for 48 h. *Escherichia coli* ATCC 11775 was used as a negative control strain of lipase activity.

Preparation of template DNA solution for PCR and REP-PCR. The bacterial cells were picked from colonies grown on tryptic soy agar (Eikenkagaku Co., Tokyo, Japan), suspended in sterile distilled water, and heated in boiling water for 10 min. After centrifugation of the suspension at $9,600 \times g$ for 10 min, the supernatant was used as the template DNA solution for PCR and REP-PCR.

PCR amplification and nucleotide sequencing. The primers were designed to detect the genes having the region corresponding to the lipase putative substrate-binding domain and to amplify this region on the basis of a multiple alignment of nucleotide sequences of the *lip*, *lipH3*, *apl-1*, *pla*, and *alt* genes (Fig. 2). The first PCR was performed with the primer pair AHL-L1 (5'-TCGGTSAARGA RAACGCYTAYG-3') and AHL-R1 (5'-VGTRKYGAACCTGAACAGRGCR TC-3'); the nucleotide symbols K, R, S, V, and Y correspond to G or T, A or G, C or G, A, C, or G, and C or T, respectively. Nested PCR was performed by using the first PCR product as the template DNA with the primer pair AHL-L2 (5'-TGAACCTGAGCTACCTGACC-') and AHL-R2 (5'-CGAACTTGAACA-GAGCATCC-3'), and this nested PCR product was used as the DNA template for the direct sequencing described below. The first PCR mixtures contained 2 μ l of 10 $\times$ EX Taq buffer (Takara Bio Co., Shiga, Japan), 1.6 μ l of 25 mM MgCl₂ (Takara Bio Co.), 1.6 μ l of 2.5 mM deoxynucleoside triphosphates (dNTPs; Takara Bio Co.), 0.24 μ l of 50 μ M primers (AHL-L1 and AHL-R1), 0.05 μ l of EX Taq (5 U/ μ l; Takara Bio Co.), 2 μ l of template DNA solution, and sterile distilled water to 20 μ l. The first PCR amplification was performed by following the touchdown PCR method program (13): preincubation at 94°C for 3 min followed by 2 cycles at 94°C for 45 s (denaturation), 62°C for 30 s (annealing), and 72°C for 90 s (extension), and then 2 identical cycles but with an annealing temperature of 60°C followed by 6 identical cycles with the annealing temperature reduced 1°C per cycle from 58°C to 53°C, 25 identical cycles with the annealing temperature at 52°C, and a final extension at 72°C for 10 min. The nested PCR mixtures contained 5 μ l of 10 $\times$ EX Taq buffer (Takara Bio Co.), 4 μ l of 25 mM MgCl₂ (Takara Bio Co.), 4 μ l of 2.5 mM dNTPs (Takara Bio Co.), 1.5 μ l of 10 μ M primers (AHL-L2 and AHL-R2), 0.25 μ l of EX Taq (5 U/ μ l; Takara Bio Co.), 5 μ l of a 10,000-fold dilution of the first PCR product as template DNA, and sterile distilled water to 50 μ l. The nested PCR amplification was performed by using the following temperature program: preincubation at 94°C for 3 min and then 35 cycles at 94°C for 30 s (denaturation), 65°C for 30 s (annealing), and 72°C for 60 s (extension); the final extension was at 72°C for 10 min. The first and nested PCR-amplified DNA fragments were confirmed by electrophoresis in 2.5% agarose gel and visualized with ethidium bromide staining.

The nested PCR product was sequenced by using a Thermo Sequenase Cy5.5 Dye terminator cycle sequencing kit (Amersham Biosciences) with primer AHL-R2. After the cycle sequencing reaction the products were analyzed on a Gene Rapid sequencer (Amersham Biosciences), and the results were analyzed by using SEQ 4 $\times$ 4 Basecaller software (Amersham Biosciences).

REP-PCR DNA fingerprints. REP-PCR was performed with the following primer pair designed by Wong and Lin (28): REP-1D (5'-NNNRCGYCGN CATCMGGC-3') and REP-2D (5'-RCGYCTTATCMGGCCTAC-3'); the nucleotide symbols M and N correspond to A and C and to A, C, G, and T, respectively. The REP-PCR mixtures contained 2 μ l of 10 $\times$ EX Taq buffer (Takara Bio Co.), 1.6 μ l of 25 mM MgCl₂ (Takara Bio Co.), 1.6 μ l of 2.5 mM dNTPs (Takara Bio Co.), 2 μ l of 10 μ M primers (REP-1D and REP-2D), 0.1 μ l of EX Taq (5 U/ μ l; Takara Bio Co.), 2 μ l of template DNA solution, and sterile distilled water to 20 μ l. The REP-PCR amplification was performed by using the following temperature program: preincubation at 94°C for 3 min and then 30 cycles at 94°C for 30 s (denaturation), 60°C for 60 s (annealing), and 70°C for 3 min (extension); the final extension was at 72°C for 16 min. REP-PCR amplification fragments were electrophoresed in 2.5% agarose gels and were photographed on a UV light transilluminator after staining with ethidium bromide.

RESULTS

Lipase activity of strains. A clear halo indicating lipase activity appeared around colonies of all *A. hydrophila*, *A.*

caviae, and *A. sobria* strains tested on tributyrin agar medium (Fig. 1). A halo did not appear around colonies of *E. coli*.

PCR amplification and nucleotide sequence analysis. The first PCR with primer pair AHL-L1 and AHL-R1 and also the nested PCR with primer pair AHL-L2 and AHL-R2 allowed amplification of DNA fragments from all *A. hydrophila*, *A. caviae*, and *A. sobria* strains tested (Fig. 2). Sequence analysis with primer AHL-R2 was used to determine the nucleotide sequence of the target region in all of the nested PCR amplified fragments. The nucleotide sequences of the amplified fragments from the various *Aeromonas* strains differed and were sorted into 38 distinct nucleotide sequence groups (Fig. 3). In the *A. hydrophila* strains, 35 distinct nucleotide sequence groups were found, and 23 of them were present in only one strain. One identical sequence group was found in three *A. caviae* strains, and two distinct sequence groups were found in three *A. sobria* strains. Although the DNA size of most of the nucleotide sequence groups in the *A. hydrophila* strains and group b-36 in the *A. caviae* strains was 111 bp, four groups (b-31, b-32, b-33, and b-34) from *A. hydrophila* strains had 108-bp fragments, and group b-35 in *A. hydrophila* and both groups b-37 and b-38 in *A. sobria* had 117-bp fragments. One of the nucleotide sequence groups (b-08) of *A. hydrophila* had a sequence identical to the corresponding region of the *pla* gene. A 30-bp nucleotide sequence encoding the amino acids V-H-F-L-G-H-S-L-G-A, which corresponds to the putative lipase substrate-binding domain in the *lip*, *lipH3*, *apl-1*, *pla*, and *alt* gene products, was found in all sequence groups. When the amino acid sequence was deduced and aligned on the basis of this consensus sequence, 15 distinct amino acid sequence groups were sorted (Fig. 4). There were 12 distinct deduced amino acid sequence groups in *A. hydrophila* strains, and 7 of the groups (aa-C, aa-E, aa-F, aa-G, aa-H, aa-K, and aa-L) were unique and were found in only one strain each. The amino acid sequence groups aa-J and aa-K, which were deduced from 108-bp nucleotide sequences, were able to align with other groups but had a 1-amino-acid deletion. Groups aa-L, aa-N, and aa-O, which were deduced from 117-bp nucleotide sequences, were able to align but had a 3-amino-acid insertion and a 1-amino-acid deletion relative to the other sequence groups. Although the amino acid sequence group aa-I was deduced from a 111-bp nucleotide sequence group, its sequence had a 1-amino-acid insertion and one deletion. In the deduced amino acid sequence alignment, the N-terminal three amino acids (positions -18 to -16) were identical in all strains tested. The C-terminal 23 deduced amino acids (positions -1 to 22), which contained the putative lipase substrate-binding domain, were also identical in all *A. hydrophila* strains, but two amino acids (positions 12 and 14) in all *A. caviae* and all *A. sobria* strains differed from those of *A. hydrophila*. The amino acid sequence groups aa-B and aa-D in *A. hydrophila* were identical to the *pla* and *alt* genes, respectively, in the deduced amino acid sequences of the region. Although the nucleotide sequences of the *apl-1*, *lipH3*, and *lip* genes were almost identical to those of the amplified fragments of the tested strains within the aligned region, the deduced amino acid sequences of these genes differed from those of the tested strains because of some deletions in the nucleotide sequences.

REP-PCR DNA fingerprints. REP-PCR was used to compare the DNA fingerprints of the tested strains. The REP-PCR

Strain No.	O sero-group	Source	Lipase activity	REP-PCR DNA fingerprints	Nucleotide sequence group	Deduced amino acid sequence group			
<i>A. hydrophila</i>									
004	11	Feces	+		b-01	aa-A			
006	11	Feces	+						
013	11	Feces	+						
057	16	Pus	+						
201	11	Feces	+		b-02	aa-A			
222	11	Feces	+						
002	11	Feces	+						
094	34	Feces	+						
010	11	Feces	+		b-03	aa-A			
224	11	Feces	+		b-04				
051	16	Feces	+		b-05	aa-B			
087	34	Feces	+						
090	34	Feces	+						
095	34	Feces	+						
061	16	Well water	+		b-06	aa-B			
003	11	Feces	+		b-07				
085	34	Feces	+		b-08	aa-B			
218	11	Feces	+		b-09				
058	16	Well water	+		b-10	aa-B			
083	34	Environmental	+		b-11				
014	11	Feces	+		b-12	aa-B			
001	11	Environmental	+						
059	16	Well water	+						
079	34	Environmental	+						
100	34	Well water	+		b-13	aa-B			
107	34	Well water	+		b-14				
049	16	Feces	+		b-15				
103	34	Well water	+		b-16				
104	34	Well water	+		b-17	aa-B			
105	34	Well water	+		b-18				
106	34	Well water	+		b-19				
062	16	Well water	+		b-20				
077	34	Environmental	+		b-21	aa-B			
096	34	Well water	+		b-22				
097	34	Well water	+		b-23				
098	34	Well water	+		b-24				
099	34	Well water	+		b-25	aa-B			
081	34	Environmental	+		b-26				
082	34	Environmental	+		b-27				
084	34	Environmental	+		b-28				
101	34	Well water	+		b-29	aa-B			
089	34	Feces	+		b-30				
007	11	Feces	+		b-31				
086	34	Feces	+		b-32				
088	34	Feces	+		b-24	aa-D			
252	16	Feces	+		b-25				
005	11	Feces	+		b-26	aa-E			
053	16	Feces	+		b-27	aa-F			
254	16	Feces	+		b-28	aa-G			
093	34	Feces	+		b-29	aa-H			
046	16	Environmental	+		b-30	aa-I			
048	16	Feces	+		b-31	aa-J			
102	34	Well water	+	b-32					
043	11	Sputum	+	b-33					
080	34	Environmental	+	b-34					
015	11	Well water	+		b-34	aa-K			
060	16	Well water	+		b-35	aa-L			
050	16	Feces	+			b-36	aa-M		
052	16	Feces	+						
<i>A. caviae</i>									
055	16	Feces	+					b-36	aa-M
056	16	Feces	+						
072	16	Pus	+		b-37	aa-N			
<i>A. sobria</i>									
008	11	Feces	+					b-37	aa-N
012	11	Feces	+						
011	11	Feces	+		b-38	aa-O			

FIG. 1. *Aeromonas* strains in this study and comparison of the REP-PCR DNA fingerprints between the nucleotide and the amino acid sequence groups. For the source of strains, all feces were obtained from patients with diarrhea except strain 014, which was isolated from feces of a healthy adult. Environmental sources included food, river water and soil, and seawater. Lipase activity was detected on a tributyrin agar medium. The low-molecular-weight fragments of the REP-PCR DNA fingerprints are shown in the left side of photographs. The details of the nucleotide sequence groups and the deduced amino acid sequence groups are shown in Fig. 3 and 4, respectively.

TABLE 1. Lipase, phospholipase, and cytotoxic enterotoxin genes cloned from *A. hydrophila* strains

Gene	Coding protein	DNA size of ORF ^a (bp)	Original strain	GenBank accession no.	Reference
<i>lip</i>	Extracellular lipase	2,253	MCC-2	U63543	9
<i>lipH3</i>	Extracellular lipase	2,052	H3	S65123	2
<i>apl-1</i>	Phospholipase C	2,052	JMP636	U14011	18
<i>pla</i>	Phospholipase A1	2,415	AH-3	AF092033	23
<i>alt</i>	Cytotoxic enterotoxin	1,104	SSU	L77573	8

^a ORF, open reading frame.

DNA fingerprints in Fig. 1 are arranged by the nucleotide sequence groups described above. The DNA fingerprinting patterns closely resembled one another between the strains sorted in the following nucleotide sequence groups: group b-14 (*A. hydrophila* 104 and 105), group b-18 (*A. hydrophila* 096, 097, 098, and 099), group b-20 (*A. hydrophila* 082 and 084), group b-30 (*A. hydrophila* 048 and 102), group b-36 (*A. caviae* 055 and 056), and group b-37 (*A. sobria* 080 and 012). The strains of other groups showed different patterns.

Source of strains, sequence groups, and DNA fingerprints.

All the strains sorted into the deduced amino acid sequence groups aa-A and aa-D were clinical isolates. Twenty-two of the 31 strains sorted into the deduced amino acid sequence group aa-B were environmental isolates, and there were only five environmental isolates sorted into the other groups. Seven of the deduced amino acid sequence groups were found in only one strain each. All of these strains were clinical isolates, but one was an environmental isolate. One clinical isolate with one environmental isolate was sorted into each of the nucleotide sequence groups b-30 and b-31 (Fig. 1).

DISCUSSION

We examined the distribution of the genes which have the region corresponding to the putative lipase substrate-binding domain by PCR in clinical and environmental isolates of *A. hydrophila* and also *A. caviae* and *A. sobria* strains belonging to serogroups O11, O16, and O34. The PCR primers were designed based on a multiple alignment of nucleotide sequences of the *lip*, *lipH3*, *apl-1*, *pla*, and *alt* genes. Therefore, the primers enabled the first PCR and subsequent nested PCR to amplify DNA fragments of the predicted size from the multiple alignment of nucleotide sequences of those genes from all *Aeromonas* strains tested. To identify the genes amplified from each strain, we sequenced all nested PCR-amplified DNA fragments. The sequenced region includes the target site that apparently corresponds to the putative lipase substrate-binding domain found in the *lip*, *lipH3*, *apl-1*, *pla*, and *alt* genes. Sequencing of the target region in the amplified DNA fragments identified nucleotide sequences corresponding to the putative lipase substrate-binding domain, and their deduced amino acid sequences were identical to the consensus sequence V-H-F-L-G-H-S-L-G-A, found in the *lip*, *lipH3*, *apl-1*, *pla*, and *alt* genes. Because lipase activity was observed on tributyrin agar medium and a region corresponding to the putative lipase substrate-binding domain was found in all strains, *A. hydrophila* and also *A. caviae* and *A. sobria* strains probably have genes coding for proteins with lipase activity. The alignment of the nucleotide sequences of the target region showed that the nucleotide sequences were sorted into 35 different groups by their sequence patterns. While the sequences of many groups were similar to each other with some substitutional nucleotides, few other sequence groups had unique sequences with either additional or fewer nucleotides in the target region. The nucleotide sequences of some of the amplified DNA fragments were

FIG. 2. Primer annealing sites and the region corresponding to the putative lipase substrate-binding domain in the nucleotide sequence alignment of the *lip*, *lipH3*, *apl-1*, *pla*, and *alt* genes. Identical nucleotides are shaded black. Primer annealing sites are marked by > or < symbols.

FIG. 3. Comparison of the nucleotide sequence groups in the amplified fragments and the reported genes: the *lip*, *lipH3*, *apl-1*, *pla*, and *alt* genes. The groups are sorted by their nucleotide sequences. The identical nucleotides are shaded black. The details of deduced amino acid sequence groups are shown in Fig. 4.

very similar to the corresponding regions of previously reported genes, indicating the presence of polymorphic sequences of the reported genes. Consequently, nucleotide sequence analysis of only the amplified fragment is not sufficient for conclusive identification of the gene. In addition, the amplified DNA fragments that have unique nucleotide sequences may be from unknown genes. Because some nucleotide substitutions, additions, and deletions were found at single or multiple loci in the sequence alignment of the amplified DNA fragments, the identification of the genes from which the DNA fragments were amplified may not be possible with PCR or restriction fragment length polymorphism analysis. Cascón et al. have reported that the *lipH3* gene was detected by PCR from all *A. hydrophila* strains included in DNA hybridization group 1 (5). However, the primers used in the PCR were designed from *lipH3* gene sequences that were highly homologous to the *lip*, *apl-1*, and *pla* genes. Therefore, the PCR with these primers probably detected the *lip*, *apl-1*, and *pla* genes as well as unreported genes instead of specifically detecting the *lipH3* gene. Granum et al. have reported that the *alt* gene was detected by PCR from all *A. hydrophila* strains tested (16), but they also used primers that may detect the *lip*, *lipH3*, *apl-1*, and *pla* genes as well as other homologous unreported genes.

When deduced amino acid sequences of the N- and C-terminal regions of the putative substrate-binding domain sequence in the target region were aligned, the deduced amino acid sequence patterns were categorized into 12 groups in *A. hydrophila* strains. The deduced amino acid sequences of the target region were identical to those of the *pla* gene and the *alt* gene in more than half and five of the strains, respectively. However, the deduced amino acid sequences in other strains were not identical to those of any reported genes. In addition, the amino acid sequences of the *lip*, *lipH3*, and *apl-1* genes differed markedly in the target region from those deduced

from the amplified DNA fragments of *Aeromonas* strains. The differences in the nucleotide sequences did not result in variations in the amino acid sequences of the target region that are identical to the corresponding region of the *pla* or *alt* gene product. However, variations in the sequence, such as nucleotide substitutions, insertions, or deletions in other regions besides the target region in this study, may result in alterations in the amino acid sequence. Therefore, sequence analysis of only the amplified target region does not allow conclusive identification of the genes as *pla* or *alt*. These findings indicate that identification of the amplified DNA fragments from the tested

FIG. 4. Comparison of the deduced amino acid sequence groups in the amplified fragments and the reported genes: the *lip*, *lipH3*, *apl-1*, *pla*, and *alt* genes. The amino acid sequences were deduced and aligned based on the sequence of putative lipase substrate-binding domain. Identical amino acids are shaded black.

strains will require complete nucleotide sequence analysis of the corresponding structural genes.

In the alignment analysis of the deduced amino acid sequences, identical sequences in all *A. hydrophila* strains were found in the N-terminal 3 and the C-terminal 23 amino acids, with the latter region containing the putative lipase substrate-binding domain sequence. These consensus sequences were also found in the groups with the unique amino acid sequences that contained two additional or one fewer amino acid than other sequences, suggesting the presence of highly conserved regions in *A. hydrophila* strains. In contrast, the deduced amino acid sequences of the amplified fragments differed markedly from those regions thought to be highly conserved in the *lip*, *lipH3*, and *apl-1* genes. Therefore, the *lip*, *lipH3*, and *apl-1* genes may be strain-specific genes of each strain in which these genes were found. The results of these alignment analyses indicate that lipase-like proteins in *A. hydrophila* have both conserved and variable regions of amino acid sequence. Since all strains showed lipase activity, these variable regions appear not to be associated with the lipase activity of the lipase-like protein in each strain. Thus, when the other regions of the gene that were not analyzed in this study are sequenced, more diversity of deduced amino acid sequence should be found among the genes, and when the number of analyzed strains is increased, more diversity should be found in the aligned sequences among the strains. Therefore, the amplified DNA fragments should sort into more nucleotide sequence groups. Accordingly, the genes from which the amplified DNA fragments came may actually be unknown genes containing nucleotide sequences that are highly homologous to the reported genes: the *lip*, *lipH3*, *apl-1*, *pla*, and *alt* genes. It is possible that the genes encoding proteins with lipase activity are classified as homologs, and distinct homologs (those with both distinctive amino acid sequence and the consensus amino acid sequence, including the putative lipase substrate-binding domain) seem to be widely distributed in *A. hydrophila*. It is interesting to consider how many distinct homologs are distributed in *A. hydrophila* and how the characteristics of the homologs may differ.

We also applied the above experimental analysis to strains of *A. caviae* and *A. sobria*. DNA fragments that contained the region corresponding to the putative lipase substrate-binding domain were PCR amplified from all three *A. caviae* and three *A. sobria* strains. The amino acid sequences deduced from the target region in the amplified DNA fragments from the *A. sobria* and *A. caviae* strains differed between the species and were not identical to those of any of the *A. hydrophila* strains. The deduced amino acid sequences of both the *A. caviae* and *A. sobria* strains were identical to that of the *A. hydrophila* strains within the N-terminal 3 and C-terminal 23 amino acids, which are the previously described highly conserved sequences, except for two amino acid substitutions. These substituted amino acids were found in the same two distinct positions in both the *A. sobria* and *A. caviae* strains, suggesting species-specific variations in the sequence. Therefore, the fragments amplified from *A. caviae* and *A. sobria* are probably gene homologs that differ from those in the *A. hydrophila* strains.

REP-PCR DNA fingerprinting was applied to examine the relationship between the genetic diversity of the PCR-amplified DNA fragments and the genetic heterogeneity in *Aeromo-*

nas strains. The results demonstrated that there was marked heterogeneity in the tested strains. A close resemblance of the DNA fingerprinting patterns was found in some strains that sorted into the same nucleotide sequence groups, and therefore the genotype may be closely related to the nucleotide sequence of the amplified fragment in these strains. However, cases in which the nucleotide sequences of the amplified DNA fragments sorted into the same nucleotide sequence groups but showed different DNA fingerprinting patterns suggest that the DNA sequence in other regions of the gene that were not analyzed in this study may differ. Therefore, additional nucleotide sequence differences are likely to be found in other regions of the gene.

Further, the relationship between the deduced amino acid sequence groups and source of strains tested was examined. More than half of the *A. hydrophila* strains were sorted into the amino acid sequence group aa-B. Twenty-two of the 31 *A. hydrophila* strains sorted into this group were environmental isolates, and there were only five environmental isolates sorted into the other groups. This finding leads to the hypothesis that the amino acid sequence of the group aa-B is identical to a region of an original amino acid sequence of the genes that encode a protein with lipase activity; therefore, these gene homologs encoding a protein with lipase activity were derived from a gene distributed among environmental strains as the origin. In addition, the clinical isolates were sorted into many distinct amino acid sequence groups, unlike the environmental isolates; moreover, six of the seven strain-specific amino acid sequence groups were found in the clinical isolates. These findings suggest that there are many gene homologs encoding a protein with lipase activity in the clinical isolates, unlike the case with the environmental isolates. This suggestion leads to the additional hypothesis that the clinical isolates of *A. hydrophila* strains are variants that were derived from the environmental *A. hydrophila* strains as a consequence of evolutionary genetic changes. One of the evolutionary genetic changes in *A. hydrophila* probably appeared as the genetic diversity among the amplified DNA fragments in this study. Besides, the clinical isolates of *A. hydrophila* possibly acquired some virulence while the genetic variations and additions involved in the evolutionary genetic changes have occurred. Some clinical isolates were sorted into the amino acid sequence group aa-B. Additionally, some clinical and environmental isolates were sorted into the same nucleotide sequence groups. These incompatibilities could be elucidated by analyzing the diversity of the nucleotide sequences of the other regions of the gene. In addition to this analyzing, the investigation of diversity of the other genes, especially virulence genes, may clarify the evolutionary relationship between the clinical and environmental isolates of *A. hydrophila*. Meanwhile, the relationship between the sources of strains and the REP-PCR DNA fingerprinting patterns was not observed. Since the genetic heterogeneity of *A. hydrophila* appears to be complicated, the genetic differences between the clinical and environmental isolates were probably hidden in the distinctive overall genetic heterogeneity of each strain.

In this study we amplified DNA fragments of genes that appear to encode proteins with lipase activity from all tested *A. hydrophila*, *A. caviae*, and *A. sobria* strains. These amplified DNA fragments are probably portions of reported or unknown

gene homologs, and the gene homologs appear to be widely distributed in the *A. hydrophila*, *A. caviae*, and *A. sobria* strains. The distinctive gene homologs from the various species are likely to differ. Additionally, the distinctive gene homologs probably differ between clinical and environmental isolates of *A. hydrophila*. In order to identify these homologs, sequencing of the complete structural genes would be necessary. Furthermore, this analysis may also clarify the relationship of the diversity in the genes to the genotyping of *Aeromonas* strains. Further investigations of the diversity in the gene homologs may be helpful to elucidate the genetic heterogeneity of these strains. In addition, analysis of homologs of other genes, especially virulence genes, may clarify the evolutionary genetic variation of *Aeromonas*.

ACKNOWLEDGMENT

This work was supported by a grant from the Scientific Research Frontier Program from the Ministry of Education, Culture, Sports, Science, and Technology.

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