THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 7, Issue of February 15, pp. 5030–5039, 2002
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Printed in U.S.A.

Discovery and Characterization of Two Isoforms of Moronecidin, a Novel Antimicrobial Peptide from Hybrid Striped Bass*

Received for publication, September 24, 2001, and in revised form, November 15, 2001
Published, JBC Papers in Press, December 5, 2001, DOI 10.1074/jbc.M109173200

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We isolated a novel 22-residue, C-terminally amidated antimicrobial peptide, moronecidin, from the skin and gill of hybrid striped bass. Two isoforms, differing by only one amino acid, are derived from each parental species, white bass (*Morone chrysops*) and striped bass (*Morone saxatilis*). Molecular masses (2543 and 2571 Da), amino acid sequences (FFHHIFRGIVHVGKTIH(K/R) LVTGT), cDNA, and genomic DNA sequences were determined for each isoform. A predicted 79-residue moronecidin prepropeptide consists of three domains: a signal peptide (22 amino acids), a mature peptide (22 amino acids), and a C-terminal prodomain (35 amino acids). The synthetic, amidated white bass moronecidin exhibited broad spectrum antimicrobial activity that was retained at high salt concentration. An α-helical structure was confirmed by circular dichroism spectroscopy. The moronecidin gene consists of three introns and four exons. Peptide sequence and gene organization were similar to pleurocidin, an antimicrobial peptide from winter flounder. A TATA box and several consensus-binding motifs for transcription factors were found in the region 5′ to the transcriptional start site. Moronecidin gene expression was detected in gill, skin, intestine, spleen, anterior kidney, and blood cells by kinetic reverse transcription (RT)-PCR. Thus, moronecidin is a new α-helical, broad spectrum antimicrobial peptide isolated from the skin and gills of hybrid striped bass.

Fish have evolved to thrive in an aqueous environment with a rich microbial flora and are presumed to use their innate immune system as the first line of defense against microbial invasion. Endogenous antimicrobial peptides (AMPs)[1] are widely distributed in nature and are considered as the earliest components in the evolution of innate immunity (1, 2). Although the primary structure of AMPs are highly heterogeneous, they can be loosely classified into three structural groups: (*a*) peptides with a disulfide-bonded β-sheet or α-helix/β-sheet (3–5), including the widespread defensins; (*b*) α-helical peptides such as the insect cecropins and the amphibian magainins (6); and (*c*) peptides with an overrepresentation of certain amino acids (proline, histidine, tryptophan, or glycine). Most AMPs share the following features: (*a*) broad spectrum antimicrobial activity against bacteria, yeast, and filamentous fungi and, for some AMPs, parasites and enveloped viruses as well; (*b*) cationic properties at physiological pH; and (*c*) an amphipathic secondary structure. Microbial killing is a consequence of the interaction of the AMP with the microbial outer membrane, which leads to membrane destabilization and channel formation. It remains unclear if channel formation alone promotes leakage of cytoplasmic contents resulting in death of the organism or if introduction of AMPs into the cytoplasm and interaction with cellular components also plays a role in microbial killing (7, 8).

Reports describe a variety of AMPs from aquatic organisms including mollusks, crustaceans, ascidians, and fishes. These include the cysteine-rich peptides of mussels (myticin) (9) and horseshoe crab (tachyplesins and polyphemusins) (10), the proline- and cysteine-rich peptides from shrimp (penaeidins) (11), and the α-helical peptides from ascidians (clavanins and styelins) (12–14) and fish (misgurin, pleurocidin, paradaxins, hagfish intestinal antimicrobial peptides, and parasin I) (15–19).

The fish AMPs, pleurocidin, paradaxin, and parasin I, have been isolated from the mucosal surface of the skin (16, 17, 19), and pleurocidin has been detected by immunolocalization in mucin granules of goblet cells in the skin and intestines (20). No fish AMPs have been previously isolated from the gill, although the huge surface area of this organ is in constant contact with a diverse array of potential pathogens in the external environment. The thin epithelial layer and abundant blood supply could provide easy access for microbes into the systemic circulation. By analogy to the mammalian airway, from which β-defensins (21) and tracheal antimicrobial peptide (22) have been isolated, we hypothesized the presence of AMPs

* This work was supported in part by the Advanced Technology Program from the Department of Commerce to Kent SeaTech Corp. and in part by CNRS and the University Louis Pasteur of Strasbourg. The Molecular Pathology Shared Resource (University of California San Diego Cancer Center) is funded in part by NCI, National Institutes of Health, Cancer Center Support Grant 5P0CA23100-16. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s)AF332621 and AF385583 (moronecidin cDNA of white bass and striped bass, respectively) and AF394243 and AF394244 (moronecidin gene of white bass and striped bass, respectively).

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[1] The abbreviations used are: AMP, antimicrobial peptide; HPLC, high performance liquid chromatography; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; cfu, colony-forming units; RP, reverse phase; ACN, acetonitrile; MIC, minimal inhibitory concentration; RT, reverse transcription; PTH, phenylthiohydantoin; wb-moronecidin, white bass moronecidin; sb-moronecidin, striped bass moronecidin; nt, nucleotide.

This paper is available on line at http://www.jbc.org

in the fish gill. We describe here our discovery and characterization of a novel α-helical AMP from the skin and gills of hybrid striped bass, which we named moronecidin. Further, we explore the potential role of this antimicrobial peptide in defense against *Streptococcus iniae*, a serious emerging pathogen of hybrid striped bass and other commercially important aquaculture species (23).

EXPERIMENTAL PROCEDURES

Tissue Collection and Purification of Antimicrobial Peptides—Adult hybrid striped bass were reared at Kent SeaTech Corp. (San Diego, CA). Skin, gill, and blood samples were harvested 12 h after bacterial challenge with an intraperitoneal injection of live *Escherichia coli* strain D22 and *Micrococcus luteus* mixture (50 μl of each organism from an overnight culture, ~10^9 cfu/ml). Tissues were immediately frozen by immersion in liquid nitrogen. Frozen samples were ground into powder with a mortar and pestle under liquid nitrogen. Proteins were extracted in 10% acetic acid supplemented with the protease inhibitor, aprotinin (1.5 μM, final concentration) by shaking on an ice-cold water bath for 3 h. After centrifugation (2800 × *g* for 20 min), the supernatants were filtered (0.45 μm, Millex™; Millipore Corp.), prepurified, and loaded onto 12-ml Sep-Pak Vac C_{18} cartridges (Waters) equilibrated with 10% acetic acid. The cartridges were washed with acidified water (0.05% trifluoroacetic acid), and two successive stepwise elutions were performed with 30 and 80% acidified acetonitrile (ACN), 0.05% trifluoroacetic acid. Both effluents were lyophilized and resuspended in water.

The 30% ACN effluents from the skin, gill, and blood extracts were subjected to reverse phase (RP)-HPLC purification through a C_{18} preparative column (10 × 220 mm; Phenomenex) on a 0–50% ACN linear gradient over 50 min (skin extract), 80 min (gill extract), and 60 min (blood extract) at a flow rate of 2 ml/min. The 80% ACN effluents from the skin and gill were purified as above, using a linear biphasic gradient of acidified ACN (0–20% over 10 min/20–80% over 50 min). Fractions were monitored for absorbance at 220 nm. Each peak was collected, lyophilized, resuspended in water, and screened for antimicrobial activity by the liquid growth inhibition assay.

Active fractions were further purified to homogeneity with a second and third round of RP-HPLC. The second purification step was performed on an analytical C_{18} column (2.5 × 220 mm; Phenomenex), using linear biphasic gradients of acidified ACN (0–15% over 10 min/15–55% over 60 min for the skin antimicrobial fractions and 0–24% over 10 min/24–44% over 80 min for the gill antimicrobial fractions) at a flow rate of 1 ml/min. The final purification step was performed on the same column as above with a linear biphasic gradient of acidified ACN from 0 to 18% over 10 min and from 18–58% over 70 min at a flow rate of 1 ml/min. After each purification step, fractions were lyophilized, resuspended in sterile water, and tested for antimicrobial activity.

Structure Determination and Microsequence Analysis—The purity of the peptides was confirmed by capillary zone electrophoresis (model 270A-HT Capillary Electrophoresis System; PerkinElmer Applied Biosystems) equipped with a fused silica tube (length, 72 cm; internal diameter, 50 μm) as described previously (24).

MALDI-TOF-MS was performed to determine the molecular masses with a Bruker BIFEXIII™ matrix-assisted laser desorption/ionization time of flight mass spectrometer (Bremen, Germany). Samples were analyzed in a linear positive mode using a "sandwich" sample preparation (25). Briefly, the sample was deposited at the surface of a thin layer of saturated solution of α-cyano-4-hydroxycinnamic acid in acetone. The drop was immediately covered with 0.5 μl of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% ACN. After air drying, the preparation was washed with 1.5 μl of 0.1% trifluoroacetic acid, dried under gentle vacuum, and analyzed. Peptide microsequencing and detection of phenylthiohydantoin derivatives were performed on a pulse liquid automatic Edman sequenator (PerkinElmer Applied Biosystems, model 473A).

General Procedure for Fmoc Solid-phase Synthesis—The amidated white bass moronecidin (wb-moronecidin) peptide was synthesized using the Fmoc strategies on a Fmoc ring amide resin according to the procedure previously described (26). Briefly, the peptide was synthesized according to classical Fmoc chemistry. Assembly of the protected peptide chain was carried out on a 25 μM scale. Following purification by solid-phase extraction and RP-HPLC using a preparative column (Aquapore RP 300 C_8, 150 × 10 mm; Brownlee™), peptide purity and identity were established by Edman degradation, capillary zone electrophoresis, and measurement by mass spectrometry.

Circular Dichroism—CD spectra of synthetic, amidated wb-moronecidin (20 μM) were measured in 20 mM potassium phosphate buffer (8 mM EDTA, pH 7.25) with or without 50% (v/v) trifluoroethanol on a CD spectrophotometer, model 202 (Aviv Instruments Inc.) with a cell path length of 0.2 mm. The CD spectra were recorded from 180 to 300 nm.

Microbial Isolates—*M. luteus* (CIPA270, gift from the Pasteur Institute Collection, Paris, France) and *E. coli*, strain D22 (an Env A1 mutant with a defect in the outer membrane, gift from P. L. Boquet, Center d'Etudes Nucléaires, Saclay, France) were used as reference strains for Gram-positive and Gram-negative bacteria, respectively. *S. iniae* K136-01 bB is an isolate from the brain of a farmed hybrid striped bass with streptococcal septicemia (Kent SeaTech Corp.). KST740ak and KSTSi6P are other *S. inaie* isolates from fish. All other bacterial isolates were from ATCC (Manassas, VA).

The fungal strains, *Neurospora crassa* (CBS 327-54) and *Fusarium culmorum* (IMI 1800420) were generous gifts from W. F. Broekaert (Université Catholique of Leuven, Belgium), *Fusarium oxysporum* (MUCL 909) from the Société Clause (France) and *Aspergillus fumigatus* from H. Koenig (Laboratory of Mycology, University of Medicine, Strasbourg, France).

Logarithmic phase cultures were used in all experiments. Bacteria and yeast were grown in Todd Hewitt broth. Filamentous fungi were grown in half-strength potato dextrose broth (Difco) supplemented with tetracycline (10 μg/ml) and cephotaxime (100 μg/ml).

Antimicrobial Assays—During the purification procedure, antibacterial activity was monitored by a liquid growth inhibition assay against *M. luteus* and *E. coli* D22, as previously described (27). Briefly, logarithmic phase bacterial cultures were diluted in the broth (1% (w/v) bactotryptone, 0.9% (w/v) NaCl) to an A_{600} of 0.001, which is approximately equivalent to 10^5 cfu/ml. Diluted bacteria (90 μl) were mixed with 10 μl of either water (control) or the RP-HPLC fraction in wells of a microtitration plate. The bacterial growth was monitored, after an overnight incubation at 25 °C, by measuring the change in the absorbance of the culture at 600 nm using a microplate reader.

The minimal inhibitory concentration (MIC) was determined as previously described (27). Briefly, bacteria, yeast, and filamentous fungi were incubated in Todd Hewitt broth in the presence of 2-fold serial dilutions of synthetic, amidated wb-moronecidin (1.25–20 μM final concentration). Bacterial growth was monitored by a liquid growth inhibition assay. MIC was expressed as a range of the highest concentration of peptide at which bacteria were able to grow and the lowest concentration that inhibited bacterial growth completely (28). To determine the minimal bactericidal concentration, medium containing peptide and organism was incubated for 18 h and then inoculated onto TH agar using a needle transfer device for a 96-well plate. Bacterial growth was assessed after overnight incubation at 37 °C.

When the effect of cations on antimicrobial activity was studied, the MIC was determined against *Staphylococcus aureus* in modified LB broth with no salt or with varying concentrations of NaCl (0–1280 mM), $MgCl_2$ (0–40 mM), or $CaCl_2$ (0–20 mM).

To determine the rate of bactericidal activity of moronecidin, the kinetic studies were performed using *S. aureus* and *Shigella flexneri*. Briefly, synthetic, amidated wb-moronecidin (3 or 6 μM) was added to a log phase culture of *S. aureus* (2 × 10^5 cfu/ml) and incubated at 30 and 37 °C. The bacterial viability was assessed at each time point (2–30 min), by plating dilutions of the bacterial suspension on Todd Hewitt agar followed by overnight incubation at 37 °C. The percentage of cfu was defined relative to the cfu obtained in the control (100% cfu at 0 min).

Hemolytic Assay—Freshly packed human or sheep erythrocytes (5 ml) were washed with phosphate-buffered saline (pH 7.4) until the supernatant was colorless and resuspended in phosphate-buffered saline (50 ml) supplemented with glucose (0.2%, w/v). Synthetic, amidated wb-moronecidin (10 μl of 800–3.125 μM, serially diluted in phosphate-buffered saline) was added to 90 μl of a 1% erythrocyte suspension (1:10 dilution of washed erythrocytes) in microcentrifuge tubes. The samples were incubated for 30 min at 37 °C and centrifuged for 10 min at 3500 rpm at room temperature. The supernatants (70 μl) were transferred to a microtiter plate, and the optical density was determined at 405 nm. The percentage of hemolysis was defined relative to the hemolysis obtained with the erythrocyte suspension treated with 0.1% SDS (100% hemolysis).

Sequence Determination of Moronecidin cDNA—RNA was extracted from the gill of an unchallenged hybrid striped bass, using TRIzol reagent (Invitrogen), according to the manufacturer's instructions. RT was performed from total RNA, using Moloney murine leukemia virus reverse transcriptase (Invitrogen) and a degenerate primer, poly(T) (see Fig. 2). The poly(T) primer (5′-CCGGAAGATCTTTTTTTTTTTTTTTT-TTTTV-3′) contains a 5′ *Bgl*II restriction site and 20 thymidine residues with a 3′-terminal V (where V represents A, C or G). A degenerate,

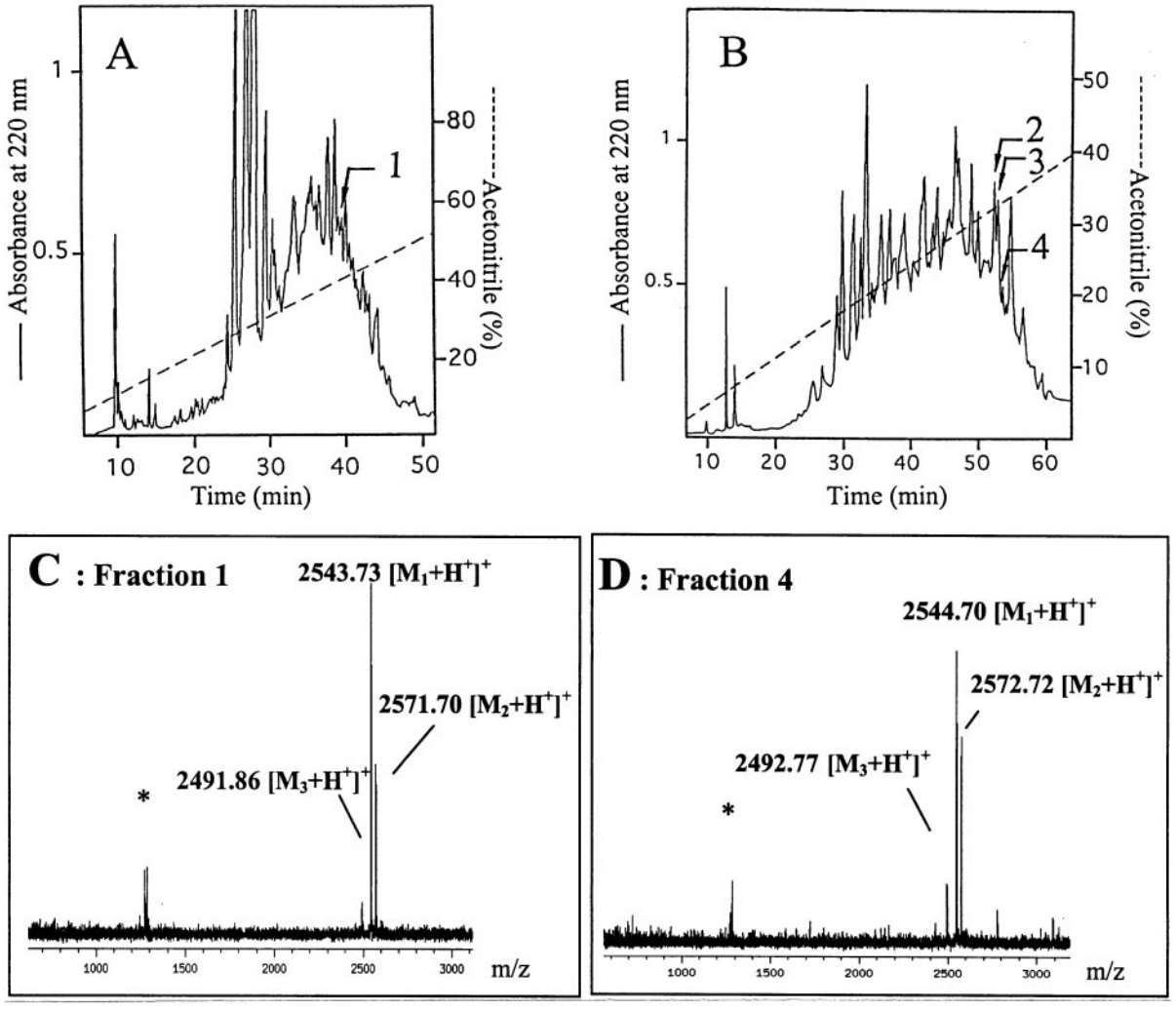

FIG. 1. *A* and *B*, purification of moronecidin from hybrid striped bass skin (*A*) and gill (*B*) by reverse phase HPLC. Acidic extracts from skin and gill of hybrid striped bass were separately prepurified by solid phase extraction on Sep-Pak. Effluents from skin and gill obtained with 30% ACN were subjected to a C_{18} preparative column on a 0–50% ACN linear gradient (*dotted line*) over 50 min (skin) and 80 min (gill) at a flow rate of 2 ml/min. Absorbance was monitored at 220 nm (*solid line*). *A*, one fraction (*peak labeled 1*) from the skin had antimicrobial activity against both *M. luteus* and *E. coli*. *B*, three fractions (*peaks labeled 2–4*) from the gill had antimicrobial activity against both *M. luteus* and *E. coli*. *C* and *D*, mass spectra obtained by MALDI-TOF-MS analysis of fractions 1 (*C*) and 4 (*D*). Values are indicated in *m/z*. The *asterisk* indicates a cluster of doubly charged ions of the three molecules (M_1, M_2, and M_3) contained in fractions 1 and 4, corresponding to the *peaks labeled* as *1* and *4* (Fig. 1, *A* and *B*), respectively.

sense primer F2 (5′-GGHATHGTYCAYGTYGGHAARAC-3′ in which R represents A + G, Y is C + T, and H is A + T + C) was deduced from the amino acid consensus sequence, GIVHVGKT, corresponding to residues 8–15 in the moronecidin mature peptide (see Fig. 2). The 3′ region of the moronecidin mRNA was determined by direct sequencing of the RT-PCR product from cDNA generated with the poly(T) primer and amplified with the primer pair F2 and poly(T) (see Fig. 2).

The 5′ region of the RNA was determined by 5′-rapid amplification of cDNA ends (29). Briefly, cDNA was synthesized with primer R1, and a "poly(A) head" was created following incubation with dATP and terminal deoxynucleotide transferase (Stratagene). The cDNA with the poly(A) head was amplified with the primer pair, 65R (see Fig. 2) and poly(T).

PCR was performed using r*Tth* DNA polymerase XL (PerkinElmer Applied Biosystems) in the GeneAmp 9600 thermocycler (PerkinElmer Applied Biosystems). The PCR products were purified from an agarose gel (1–2%) using QiaQuick gel purification kit (Qiagen) and directly sequenced by using a PCR primer and the Applied Biosystems BigDye terminators.

Sequence Determination of Moronecidin Genomic DNA—DNA was extracted from the skin of striped bass and white bass using DNAzol (Molecular Research Center, Inc.), according to the manufacturer's instructions. A PCR product was generated by amplifying DNA with the primer pair 8F and R1 (see Fig. 2). The 3′- and 5′-flanking sequences were determined by inverse PCR (30). Briefly, DNA digested with *Xba*I or *Dra*I was intramolecularly ligated (T4 ligase, Promega) and amplified by the primer pair 65R and 86F (see Fig. 2). Amplification and sequence determination of the PCR products were performed as described above.

Bacterial Challenge of White Bass and Gene Expression—Eight white bass fingerlings (20–30 g) were immersed in either a suspension of the fish pathogen, *S. iniae* K136-01 bB (1.33×10^8 cfu/liter) or sterile diluted Todd Hewitt broth (control) for 2 min. Three challenged and three mock-challenged fingerlings were randomly selected, anesthetized, and sacrificed 27 h postchallenge. Tissue samples for mRNA analysis (10–100 mg of skin, gill, intestine, liver, spleen, anterior kidney, and whole blood) were immediately homogenized in TRIzol. Brain tissue from each fish was plated on blood agar (tryptic soy agar plus 5% sheep blood) to detect infection with *S. iniae*. The remaining five challenged and five mock-challenged fish were monitored for mortality for 7 days postchallenge, and brain tissue from deceased fish were also tested for *S. iniae*.

To determine the site and inducibility of moronecidin gene expression, moronecidin mRNA was quantitated in each tissue sample, by kinetic RT-PCR (31). Moronecidin cDNA and cDNA from ribosomal 18 S RNA were synthesized using primers 65R and 18S-R in the same tube and quantitated by kinetic PCR using SYBR Green PCR Master Mix (PerkinElmer Applied Biosystems) and the GeneAmp 5700 thermocycler (PerkinElmer Applied Biosystems). A primer pair, 331F and 65R, was designed to span an intron and preferentially amplify moronecidin

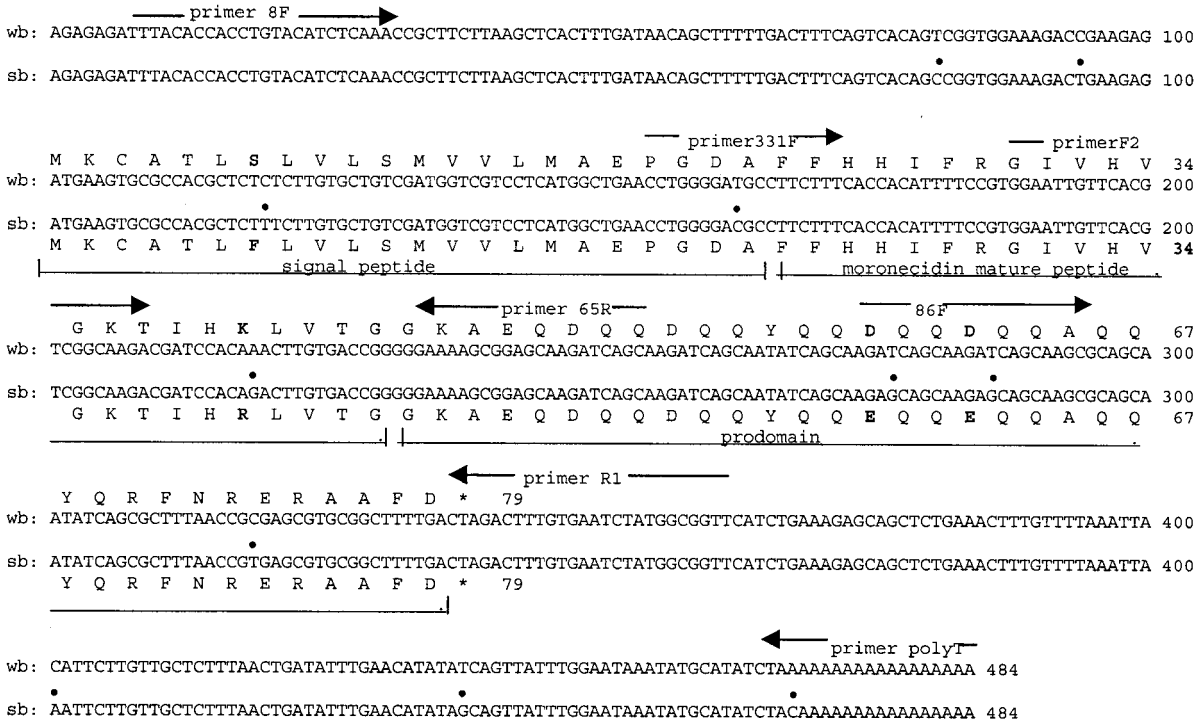

FIG. 2. **Nucleotide and predicted amino acid sequence of wb- and sb-moronecidin cDNA.** Sequencing revealed 11 loci with single-nucleotide differences (indicated by *dots*), which resulted in four amino acid changes (shown in *boldface type*). Binding sites for primers are shown with *arrows* (5′ to 3′). The organization of the peptide domains (signal peptide, mature peptide, and prodomain) is shown by the *bars*. The stop codon is indicated with an *asterisk*.

cDNA (amplicon size 99 bp) and not genomic DNA (amplicon size 763 bp). A primer pair, 18S-F (5′-GTTCGATTCCGGAGAGGGAG-3′) and 18S-R (5′-CCTTCCTTGGATGTGGTAGCC-3′), was designed from the 51-bp conserved region of yeast, plant, and mammalian 18 S rRNA genes. Standard curves for the kinetic PCR of moronecidin and 18 S cDNA were generated by amplification of serial dilutions of cDNA prepared from a challenged fish gill. Quantitation of moronecidin and 18 S cDNA for all samples was derived from the standard curves and expressed as relative units. The arbitrary units of moronecidin were normalized by the arbitrary units of 18 S for each sample.

Computer Analysis—The open reading frame was predicted using the BCM Search Launcher (available on the World Wide Web at dot.imgen.bcm.tmc.edu:9331/seq-util/seq-util.html). The Shiffer-Edmundson helical wheel diagram was predicted by using Protean 3.05 Molecular Biology Applications DNASTAR f.

Protein masses, isoelectric points, and aliphatic index were predicted using the Atelier Bioinformatique Web site (www.up.univ-mrs.fr/~wabim/d_abim/compo-p.html). The hydropathy profile of peptides was calculated by the Kyte-Doolittle method over a window of 7, using the Protein Hydrophilicity/Hydrophobicity Search and Comparison Server at the Bioinformatics Unit, Weizmann Institute of Science, Israel (bioinformatics.weizmann.ac.il/hydroph/). The presence and location of signal peptide cleavage sites in amino acid sequences were predicted using the SignalP World Wide Web server (www.cbs.dtu.dk/services/SignalP) (32). Nucleotide sequence comparison between white bass and striped bass was performed using a pairwise sequence alignment (searchlauncher.bcm.tmc.edu:9331/seq-search/alignment.html). A homology search was performed using BLASTP 2.1.2 and TBLASTN 2.1.3, in Blast 2 sequence similarity search by the Genome Net WWW Server (www.genome.ad.jp) (33). Putative transcription factor binding sites were predicted by MatInspector/TRANSFAC, BCM Search Launcher: Gene Feature Searches (searchlauncher.bcm.tmc.edu:9331/seq-search/gene-search.html) (34).

RESULTS

Purification and Primary Structure of Moronecidin—Skin, gills, and blood from challenged fish were extracted under acidic conditions and separately prepurified by solid phase extraction onto Sep-Pak. The fractions obtained after elution with 30 and 80% ACN were analyzed by RP-HPLC, and the HPLC fractions were tested for antimicrobial activity. While no activity was found in the HPLC fractions obtained from the 80% Sep-Pak eluate (data not shown), a total of four HPLC fractions from the 30% ACN Sep-Pak eluate from the skin (one fraction) and gills (three fractions), but not blood, had antimicrobial activity (*peaks labeled 1–4*, Fig. 1, *A* and *B*). These four fractions were active against both *M. luteus* and *E. coli* D22. In the present study, we focused our attention on the fractions labeled *1* and *4* from the skin and gill extracts, respectively. Fractions 1 and 4 were further purified to apparent homogeneity by two additional analytical RP-HPLC purification steps (data not shown). MALDI-TOF-MS analysis of fraction 1 (skin) and fraction 4 (gill) revealed, in both cases, two different molecules with molecular masses at ~2543 Da (2543.73 M_1H^+ and 2544.70 M_1H^+ in fractions 1 and 4, respectively) and 2571 Da (2571.70 M_2H^+ and 2572.72 M_2H^+ in fractions 1 and 4, respectively) (see Fig. 1, *C* and *D*). A common contaminant ~2491 Da (2491.86 M_3H^+ and 2492.77 M_3H+ in fractions 1 and 4, respectively) was observed in both fractions. The mass differences observed between the three molecules from fraction 1 (Fig. 1*C*) and the three molecules from fraction 4 (Fig. 1*D*) were not considered significant, but rather resulted from experimental variations (*i.e.* calibration) based on the mass variations for the control peptides (data not shown). This interpretation was further confirmed by capillary zone electrophoresis.

Fraction 1 (skin) was subjected to microsequencing by Edman degradation. Unique phenylthiohydantoin (PTH)-derivative signals were observed in all of the Edman degradation cycles except for cycle 18, in which two PTH-derivative signals (PTH-Arg and PTH-Lys) were observed. The mass difference between an arginine residue (175 Da) and a lysine residue (147 Da) perfectly matched the 28-Da mass difference between the two molecules (2571 and 2543 Da). Thus, the two molecules were assumed to correspond to peptide isoforms differing by a single residue at position 18 (arginine or lysine). We named the antimicrobial peptide "moronecidin" after the genus of the fish. Because the measured mass (2571 and 2543 Da) did not match

the calculated mass of 19 amino acids identified by sequencing (2314.8 Da and 2286.8 Da), we assumed that the peptide sequences were incomplete. In order to obtain the full peptide sequence, the cDNA sequence was determined.

Moronecidin cDNA Sequence—The complete sequence for the moronecidin cDNAs was determined from RNA from the gill of unchallenged, hybrid striped bass (Fig. 2). Analysis of the cDNA revealed two sequences with single-nucleotide differences at 11 loci, which resulted in four changes in the predicted amino acid sequence. Analysis of genomic DNA extracted from white bass and striped bass confirmed two distinct sequences that differed at these 11 loci. Thus, hybrid bass contain two isoforms of moronecidin, wb-moronecidin and striped bass moronecidin (sb-moronecidin), from each parental strain. The moronecidin cDNAs were 466 nt for white bass (GenBank™ accession number AF332621) and 468 nt for striped bass (GenBank™ accession number AF385583), exclusive of the poly(A) tail.

Both cDNAs had an open reading frame of 270 bases with a coding capacity of 79 amino acids, which contained the mature moronecidin sequence. Three methionine codons (nt positions 101, 134, and 146 of cDNA) were identified upstream of the mature peptide sequence. The first methionine codon (nt position 101) is most likely to be the translation start site, because it provides a typical signal peptide motif with a basic residue (lysine) followed by a hydrophobic region. Comparison of the predicted amino acid sequences based on the cDNA sequences and the two measured masses suggested that three terminal amino acids were missing from the 19-residue N-terminal sequence obtained after Edman degradation. The calculated masses of the predicted 22-residue isoforms of the mature peptide, 2544.08 Da (wb-moronecidin; FFHHIFRGIVHVGKTIHKLVTG) and 2572.10 Da (sb-moronecidin; FFHHIFRGIVHVGKTIHRLVTG), matched the measured masses with a difference of 1 Da (measured masses smaller than predicted masses). This discrepancy (1 Da) suggests a possible amidation of the C-terminal glycine (position 22 of the mature peptide). This proposed amidation is further supported by the presence of an extra glycine residue (position 1 in prodomain) adjacent to the C-terminal glycine residue of the mature peptide. Thus, moronecidin prepropeptide is predicted to consist of three domains: (i) a hydrophobic signal peptide (22 amino acids), (ii) a mature peptide (22 amino acids), and (iii) a C-terminal prodomain (35 amino acids). A predicted cleavage site for the hydrophobic signal peptide coincided with the amino terminus of the mature peptide. The signal peptides of the two isoforms differed by only one amino acid (serine or phenylalanine). This amino acid substitution did not affect the calculated hydrophilicity or isoelectric point (pI 4.05) of the two signal peptides, which are hydrophobic and negatively charged. The putative C-terminal prodomain of the two isoforms differed by two amino acids, and both contained six repeats of the motif *X*QQ (where *X* represents Asp, Tyr, Glu, or Ala) (Fig. 2). Both prodomains are hydrophilic and negatively charged (pI 4.05 and 4.21 for white bass and striped bass, respectively).

Genomic Organization of Moronecidin—The nucleotide sequence for the moronecidin gene was determined for white bass (GenBank™ accession number AF394243; Fig. 3) and striped bass (GenBank™ accession number AF394244). The genomic organization was similar in the two fish species (Fig. 4). Both moronecidin genes consist of three introns and four exons. The 5′-untranslated region extends from exon 1 (99 bp) through the first nucleotide of exon 2. The signal peptide is encoded by exon 2 (22 bp). The mature peptide is encoded by exon 2 (34 bp), exon 3 (19 bp), and exon 4 (13 bp). The prodomain and 3′-untranslated region are both encoded by exon 4. A canonical polyadenylation signal was found in the 3′-untranslated region.

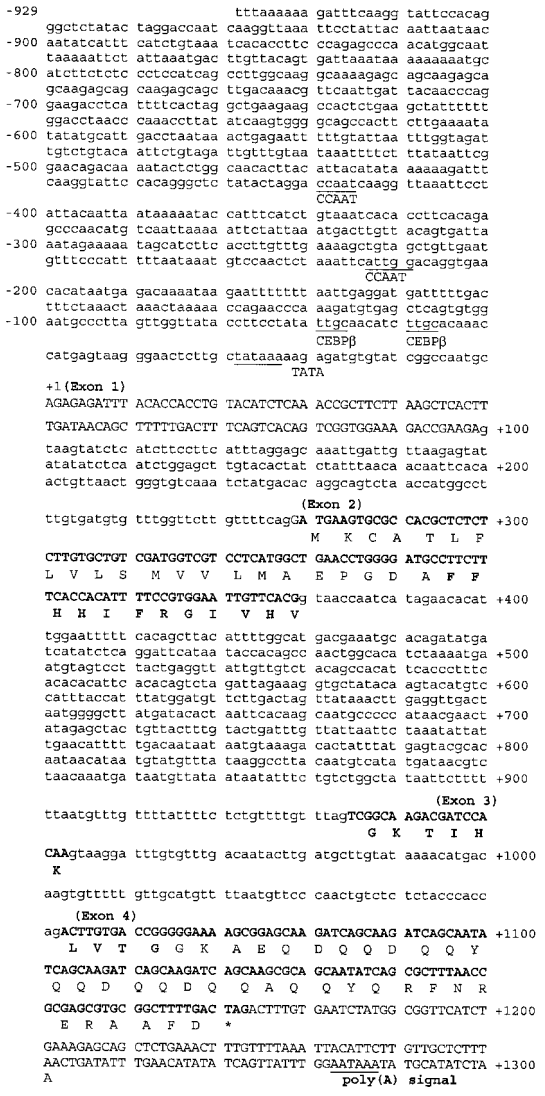

FIG. 3. **Nucleotide sequence of white bass moronecidin genomic DNA and predicted amino acid sequence.** Exons (*capital letters*), coding sequence (*boldface capital letters*), upstream sequence, and introns (*lowercase*) were determined by comparison with the cDNA sequence. The amino acid sequence of the mature peptide is shown in boldface type. Nucleotide positions are numbered from the transcription start site (+1). Consensus binding motifs for transcription factors, the TATA box, and polyadenylation signal are *underlined*.

The upstream sequences of the moronecidin genes were almost identical between white bass DNA and striped bass DNA, with the exception of a 369-bp additional sequence in striped bass DNA inserted ~500 bp upstream of the transcriptional start (Fig. 4). Within 500 bp upstream of the transcriptional start site, several putative binding sites for transcription factors were predicted (MatInspector). A TATA box was identified in the typical position, 29 bp upstream of the transcriptional start site. Two putative CCAAT boxes were found in sense and antisense orientation (nt positions relative to the transcriptional start site: −420 and −210). Two putative binding sites for CCAAT/enhancer-binding protein β in antisense orientation were found (nt positions −67 and −57).

Secondary Structure of the Mature Peptide—The amino acid sequences of wb- and sb-moronecidin mature peptides differ by one amino acid at position 18 (lysine *versus* arginine). Both sequences are rich in cationic residues (7 of 22, 33%) with

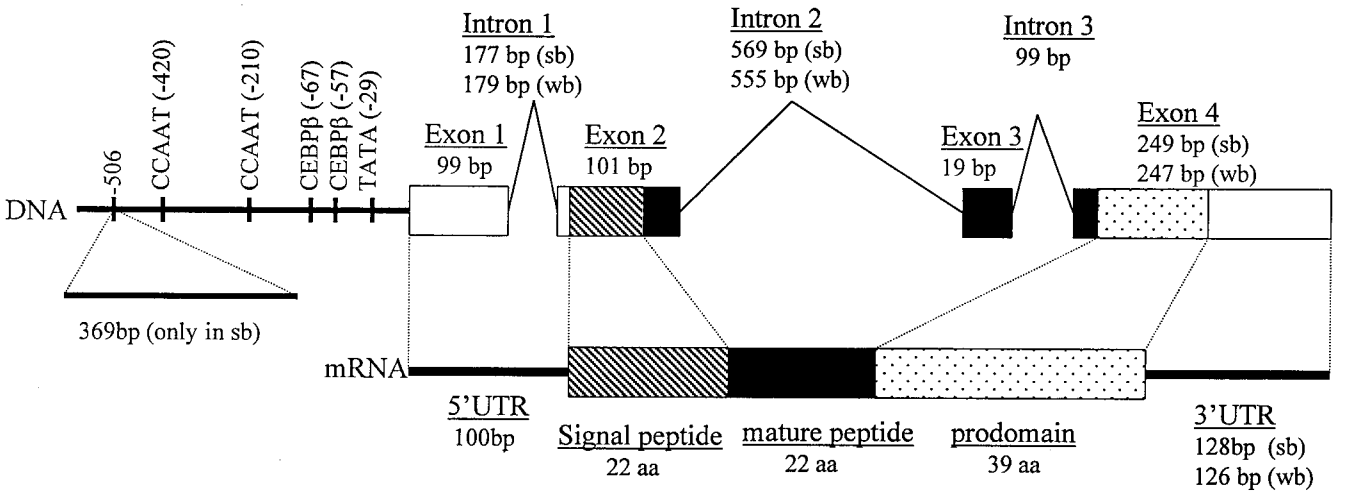

FIG. 4. **Organization of white bass and striped bass moronecidin genes, mRNA, and upstream region.** *CEBPβ*, CCAAT enhancer-binding protein.

predicted pI values of 11.60 and 12.40, respectively. In addition, the presence of a C-terminal amidated glycine residue contributes additional positive charge to the mature peptide.

Shiffer-Edmundson helical wheel modeling of the wb- and sb-moronecidin mature peptides revealed clustering of hydrophobic (leucine, glycine, valine, isoleucine, and phenylalanine) and hydrophilic/basic (arginine, lysine, and histidine) residues on opposing sides of the helical wheel (Fig. 5*A*). This result suggests an α-helical structure for the mature moronecidins.

This projection was verified by CD spectroscopy of synthetic, amidated wb-moronecidin in the presence or absence of trifluoroethanol. A standard α-helical signal was detected in the presence of 50% trifluoroethanol, while an unordered signal was detected without trifluoroethanol (Fig. 5*B*). Thus, the α-helical conformation of wb-moronecidin was confirmed in the presence of a structure-promoting solvent.

Antimicrobial Spectrum, Kinetics, Salt Sensitivity, and Hemolytic Activity of White Bass Moronecidin—The antimicrobial spectrum was determined using synthetic, amidated wb-moronecidin (Table I). The peptide was active against all Gram-positive bacteria tested (MIC <20 μM), and showed especially strong activity against methicillin-resistant *S. aureus* and all of the streptococcal strains tested including two isolates of the fish pathogen, *S. iniae* (MIC 1.25–2.5 μM). Most of the Gram-negative bacteria were sensitive to less than 20 μM moronecidin with the exception of *Aeromonas hydrophila*, *Neisseria gonorrhea*, and *Serratia marcescens* (MIC > 20 μM). The minimal bactericidal concentration for all organisms tested was either equal to or twice the MIC. Among the filamentous fungi tested, all were sensitive to moronecidin above ~3 μM (Table I), with the exception of *A. fumigatus* (MIC 50–100 μM). All of the yeast strains tested were sensitive to 10–20 μM moronecidin.

In the kinetic study, we used two highly sensitive bacterial strains, *S. aureus* and *S. flexneri*, to evaluate bactericidal activity of synthetic, amidated wb-moronecidin. Bacterial killing was time-, dose-, and temperature-dependent (Fig. 6). Within 1 min, 90% of the *S. aureus* were killed by the incubation with 6 μM (2 times the MIC; see Table I and Fig. 6) moronecidin at 37 °C, whereas 10 min were required at half that concentration (MIC 3 μM). Interestingly, a lower temperature (30 °C) reduced the rate of killing at both peptide concentrations (3 or 6 μM). Similar results were observed with *S. flexneri* (data not shown). This may suggest superior antibacterial activity at temperatures that promote more rapid bacterial growth.

We also explored the effect of cations, which may interfere with the interaction of positively charged moronecidin and the

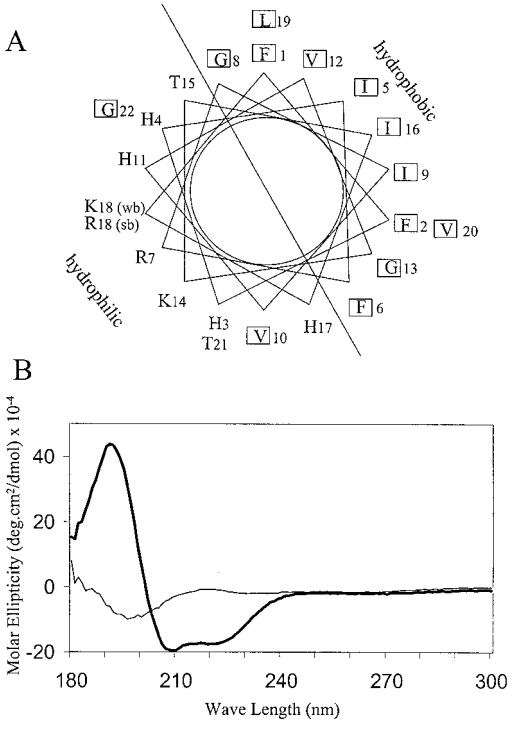

FIG. 5. *A*, helical wheel diagram projecting an amphipathic α-helical conformation of white bass and striped bass moronecidins. *Boxes* indicate hydrophobic amino acids. Residues are numbered starting from the N terminus of the mature peptide. *B*, circular dichroism spectrum for synthetic, amidated white bass moronecidin. The spectra were obtained in 20 mM potassium phosphate buffer (8 mM EDTA, pH 7.25) in the presence (*thick line*) or absence (*thin line*) of 50% (v/v) trifluoroethanol.

negatively charged microbial surface. MICs of synthetic, amidated wb-moronecidin against *S. aureus* were unchanged in the presence of up to 80 mM NaCl and only doubled in the presence of 160–1280 mM NaCl (Table II). However, in the presence of divalent cations, a 2-fold increase in the MIC values was observed between 1 and 20 mM $MgCl_2$ and between 1 and 5 mM $CaCl_2$. A 4-fold increase in the MICs was recorded in the presence of 40 and 10 mM $MgCl_2$ and $CaCl_2$, respectively.

Synthetic, amidated moronecidin was not hemolytic for human or sheep red blood cells at concentrations below 2.5 μM (Table III), a concentration highly active against many of the microorganisms tested. Hemolytic activity was observed above 5 μM in a dose-dependent manner.

TABLE I

Antimicrobial spectrum of synthetic, amidated wb-moronecidin

ATCC#, American Type Culture Collection number; MBC, minimal bactericidal concentration; NA, not applicable; VRE, vancomycin-resistant enterococcus; MRSA, methicillin-resistant *S. aureus*; *S. iniae* KST strains, isolates from hybrid striped bass (Kent SeaTech Corp., San Diego, CA). The highest concentration tested was 20 μM, except for filamentous fungi (100 μM). MICs marked with an asterisk were confirmed in an independent experiment.

Microorganisms	ATCC#	MIC	MBC
		μM	μM
Gram-positive bacteria			
E. faecalis (*VRE*)	51299	5–10	5–10
E. faecalis	29212	2.5–5	5–10
Listeria monocytogenes	7616	2.5–5	2.5–5
M. luteus	49732	10–20	10–20
S. aureus (MRSA)	33591	1.25–2.5*	1.25–2.5
Staphylococcus epidermidis	12228	5–10	5–10
Staphylococcus saprophiticus	49907	5–10	5–10
Staphylococcus xylosus	49148	>20	>20
Streptococcus agalactiae	12386	1.25–2.5	1.25–2.5
Streptococcus bovis	49147	1.25–2.5	1.25–2.5
Streptococcus equisimilis	12388	2.5–5	5–10
Streptococcus mitis	6249	1.25–2.5	2.5–5
Streptococcus pneumoniae	49619	1.25–2.5	5–10
Streptococcus pyogenes	19615	1.25–2.5	2.5–5
S. iniae, KST740ak	NA	1.25–2.5*	2.5–5
S. iniae, KSTSi 6P	NA	1.25–2.5*	2.5–5
Gram-negative bacteria			
A. hydrophila	35654	>20	>20
Burkholderia cepacia	17765	>20	>20
Vibrio cholera	10957	2.5–5*	2.5–5
E. coli	25922	5–10*	10–20
E. coli	35150	5–10	10–20
Enterobacter cloacae	35030	10–20	10–20
Enterobacter aerogenes	35029	10–20	10–20
Klebsiella pneumoniae	10031	2.5–5	5–10
Klebsiella oxytoca	49131	5–10	5–10
Moraxella catarrhalis	25340	2.5–5*	2.5–5
N. gonorrhea	43069	>20	>20
P. aeruginosa	35032	5–10*	10–20
Salmonella choleraesuis	14028	10–20*	10–20
Salmonella typhimurium	13311	10–20*	10–20
Salmonella arizonae	13314	10–20	10–20
S. marcescens	8100	>20	>20
S. flexneri	12022	2.5–5	5–10
Shigella sonnei	9290	5–10	10–20
Yersinia enterocolitica	23715	2.5–5	2.5–5
Filamentous fungi			
N. crassa	NA	1.56–3.12	NA
A. fumigatus	NA	50–100	NA
F. oxysporum	NA	0.78–1.56	NA
F. culmorum	NA	0.39–0.78	NA
Yeast			
Candida albicans	66027	10–20	NA
Candida glabrata	66032	10–20	NA
Candida lusitania	66035	10–20	NA
Candida tropicalis	66029	10–20	NA

Moronecidin mRNA Expression—In fish challenged or mock-challenged with *S. iniae*, the level of moronecidin mRNA quantitated by kinetic PCR was relatively high in the gill, intestine, spleen, anterior kidney, and blood; low in skin; and virtually undetectable in liver (Fig. 7). Although increased expression of mRNA following bacterial challenge was observed in gill, spleen, anterior kidney, and blood, only a 4-fold or greater difference in the kinetic PCR result is considered significant with our method (31). Therefore, significant differences were not observed in the level of moronecidin gene expression following *S. iniae* challenge with our protocol. Our protocol for challenging fingerlings with *S. iniae* resulted in the death of three of five challenged and none of five mock-challenged fingerlings during 7 days of observation. *S. iniae* was recovered from the brains of five of eight challenged fingerlings. Under these conditions, we did not detect significant inducible expression of moronecidin.

DISCUSSION

Moronecidin is a 22-amino acid peptide that belongs to the amphipathic α-helical family of AMPs. This novel AMP isolated from the skin and gill of hybrid striped bass exists in two isoforms, one from each parental species. Interesting properties of moronecidin include its presence in the gills, its high histidine content, its broad antimicrobial spectrum including filamentous fungi and yeast, and its relatively salt-tolerant antimicrobial activity.

α-Helical AMPs are widely distributed across diverse phyla, from insects to mammals. Similarities to mature moronecidin were found in many other α-helical AMPs, such as pleurocidin (from the skin and intestine of winter flounder, *Pleuronectes americanus*), ceratotoxins (from the female reproductive accessory glands of the medfly, *Ceratitis capitata*), dermaseptins (from skin of the arboreal frog, *Phyllomedusa bicolor*), hagfish

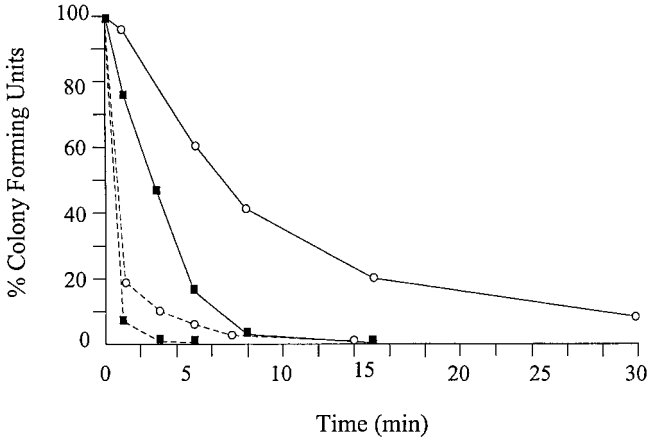

FIG. 6. **Kinetics of *S. aureus* killing by moronecidin.** Synthetic, amidated white bass moronecidin (3 μM, *solid lines*; 6 μM, *dotted lines*) was added to a log phase culture of *S. aureus* (2×10^5 cfu) at 30 °C (*circles*) or 37 °C (*squares*). Aliquots were plated on LB agar at various time points, and cfu were determined after overnight incubation at 37 °C. The percentage of cfu was defined relative to the cfu obtained in the control (100% at 0 min). Each point represents the average of two independent experiments.

TABLE II
Effect of mono- and divalent cations on moronecidin activity against S. aureus

MIC was determined using synthetic, amidated wb-moronecidin with varying concentration of salts. The results of two independent experiments (Expt.) are shown. ND, not determined.

Salt	Concentration	MIC	
		Expt. 1	Expt. 2
	mM	μM	
NaCl	0	1.25–2.5	1.25–2.5
	40	1.25–2.5	1.25–2.5
	80	1.25–2.5	1.25–2.5
	160	2.5–5	2.5–5
	320	2.5–5	2.5–5
	640	2.5–5	2.5–5
	1280	ND	2.5–5
$MgCl_2$	1	1.25–2.5	1.25–2.5
	5	2.5–5	2.5–5
	10	2.5–5	2.5–5
	20	2.5–5	2.5–5
	40	ND	5–10
$CaCl_2$	1	1.25–2.5	1.25–2.5
	5	2.5–5	2.5–5
	10	5–10	2.5–5
	20	10–20	5–10

intestinal antimicrobial peptides, and clavanins and styelins (from the hemocytes of the ascidian, *Styela clava*) (Fig. 8*A*).

Both wb-moronecidin and sb-moronecidin are rich in basic amino acids, which accounts for the high net positive charge of the molecule. The net positive charge of moronecidin is expected to be even greater with the predicted C-terminal amidation. Although C-terminal amidation has been reported for many α-helical AMPs from insects (melittins and cecropins), arachnids (lycotoxins), chordates (clavanin), amphibians (dermaseptins, caerins), and mammals (cathelicidins) (1), moronecidin is the first reported example of an amidated AMP from fish.

Pleurocidin prepropeptide shares remarkable homology with moronecidin prepropeptide (20) (Fig. 8, *B* and *C*). The conserved region extends from the N-terminal signal peptide (77% similarity and 41% identity) through the mature peptide (63% similarity and 27% identity). The genomic organization is also conserved between the two peptides (Fig. 8*B*), strongly suggesting an evolutionary relationship between the two genes. A

TABLE III
Hemolytic activity of moronecidin

The hemolytic assay was performed using synthetic, amidated wb-moronecidin and either sheep or human erythrocytes. The average of two independent experiments is shown.

Concentration	Hemolysis of erythrocytes	
	Human	Sheep
μM	%	%
0.31	0	0
0.63	0	0
1.25	0	0
2.5	1	0
5	19	0
10	55	11
20	100	51
40	100	80
80	100	100

previous attempt to find pleurocidin-related genes in other fish species by Southern hybridization using pleurocidin genomic probes detected related genes only among flatfish (35). However, discovery of moronecidin demonstrates that pleurocidin-like AMPs exist in a broader range of fish. Divergence in codon usage between the flat fish and other fish species may account for the previous failure to detect pleurocidin-related genes by Southern hybridization.

Despite the amino acid similarities of moronecidin with the mature ceratotoxins and dermaseptins (Fig. 8*A*), their prepropeptides and genes have a different organization (36, 37). In both prepropeptides, the propieces are located on the N-terminal side of the mature peptide. In addition, dermaseptin genes differ from moronecidin genes by having only two exons and one intron. Thus, these genes are not likely to be evolutionarily related.

Moronecidin and clavanins are both histidine-rich α-helical AMPs (4 histidines of 22 residues for wb- and sb-moronecidins and 4 histidines of 23 residues for clavanin A) (12) (Fig. 8*A*). Clavanins are unusual AMPs in that their cationicity derives primarily from histidines rather than from arginine or lysine residues. Clavanin A is active at pH 5.5 but relatively inactive at pH 7.4 (38). Comparison of the native clavanin A and the synthetic variant clavanin AK (four histidine $\rightarrow$ lysine substitutions) has shown that the histidine residues in clavanin A confer pH-dependent antimicrobial activity. While the intravacuolar pH of ascidian hemocytes is controversial, it is generally agreed to be acidic (39), which would preserve the activity of the peptide. The antimicrobial activity of wb-moronecidin was only tested at neutral pH. The greater positive net charge of moronecidins compared with clavanin A (calculated pI 8.75) may account for the antimicrobial activity of wb-moronecidin at neutral pH.

An unusual property of wb-moronecidin is its salt-tolerant antimicrobial activity. The synthetic peptide inhibited the growth of *S. aureus* at sodium chloride concentrations up to 1280 mM, which is roughly equivalent to the salt concentration of sea water (~1 M sodium chloride). The genus *Morone* includes species that inhabit both marine and freshwater environments. Clavanins and styelins, both from marine organisms, also retain antimicrobial activity in the presence of high salt (up to 100 mM sodium chloride for clavanin A and 400 mM for styelin A and B) (13, 38). Thus, AMPs from fish and marine invertebrates may have evolved to function in habitats with a wide variation in salt concentration.

Synthetic, amidated wb-moronecidin exhibited broad antimicrobial activity against fungi, yeast, and Gram-positive and Gram-negative bacteria, including antibiotic-resistant bacteria, such as *Pseudomonas aeruginosa*, methicillin-resistant *S. aureus*, and vancomycin-resistant *Enterococcus faecalis*. Syn-

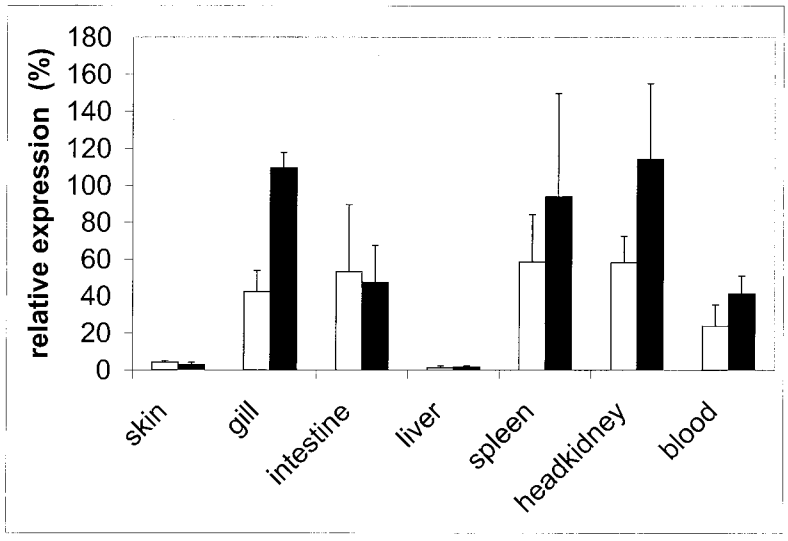

FIG. 7. **Moronecidin mRNA expression.** White bass fingerlings were challenged with *S. inaie* (*black bar*, $n = 3$) or mock-challenged (*white bar*, $n = 3$). Moronecidin mRNA and 18 S mRNA from multiple organs were quantitated by kinetic RT-PCR. Moronecidin mRNA level was normalized by the 18 S mRNA level. The average mRNA level in each tissue was expressed as a percentage of the average mRNA level in the gills of one challenged fish (100%).

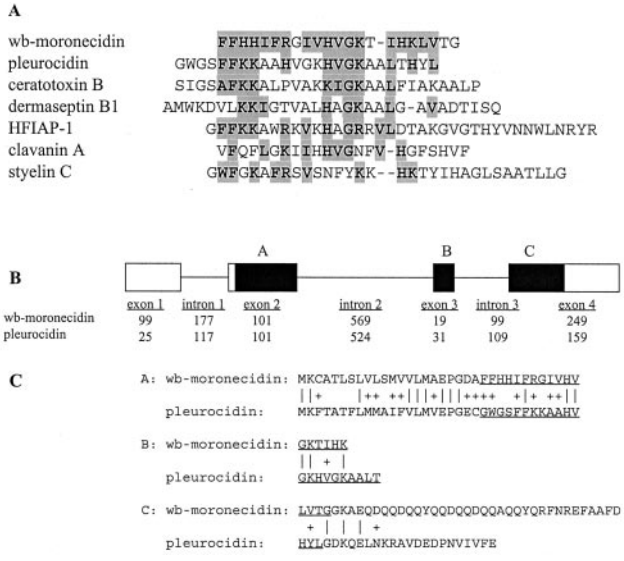

FIG. 8. *A*, amino acid sequence similarity between moronecidin and other antimicrobial peptides. Identical or similar amino acid residues are *shaded*. *HFIAP-1*, hagfish intestinal antimicrobial peptide-1. *B* and *C*, schematic representation of the genomic organization (*B*) and amino acid sequence alignment (*C*) of white bass moronecidin and winter flounder pleurocidin. *Black boxes A*, *B*, and *C* correspond to the coding sequences in exons 2, 3, and 4, respectively. The mature peptides are *underlined*. Amino acid identity (*vertical lines*) and similarity (+) are shown between the two peptides.

thetic, amidated wb-moronecidin also demonstrated activity against pathogenic isolates of *S. iniae*, which is an important pathogen for farmed hybrid striped bass, tilapia, and other aquaculture species (23). The MIC of moronecidin against *S. iniae* (1.25–2.5 μM) is similar to the MICs of β-lactam antibiotics against this organism (40). In our *in vitro* experiments, we did not demonstrate resistance of this organism to moronedicin. The fact that challenged fish died, despite the efficacy of moronecidin against *S. iniae*, emphasizes that AMPs alone are not sufficient for protection against infection.

The results of kinetic RT-PCR analysis demonstrated constitutive expression of moronecidin mRNA in multiple tissues, especially gill, intestine, spleen, anterior kidney, and blood. High expression in the gill and isolation of the active peptide from this organ suggest that both synthesis and maturation of the peptide occur at this tissue site. This is the first reported AMP to be isolated from the gill. All previously characterized fish AMPs, including pleurocidin (winter flounder), hagfish intestinal antimicrobial peptides, misgurin (mudfish), pardaxin (sole), and parasin (catfish), were found in cutaneous or mucosal sites. Given that the gill, like the lung in terrestrial animals or the trachea in insects, is an important contact point with the external environment, it is not surprising that this organ should be well protected by a variety of host defense mechanisms.

Kinetic PCR analysis showed that the number of mRNA transcripts in the skin was significantly lower than in the gill. However, the amount of moronecidin mature peptide at these sites is unknown. A moronecidin antibody would provide a tool to quantify the peptide in different tissues. One factor that complicates the correlation between levels of mRNA and mature peptide is that low rates of constitutive peptide synthesis coupled with concentration of stable peptide in mucous-secreting cells could result in accumulation of peptide. Second, moronecidin could be synthesized in other tissues and transported in the blood to the skin, where it is sequestered by an unknown mechanism and stored for eventual release. Finally, the correlation between levels of message and peptide may be further complicated by gene duplication, which is well documented for many AMPs (*e.g.* dermatoxins and ceratotoxins) (41, 42). Additional moronecidin genes may exist whose mRNA was not detected by our moronecidin-specific primer pairs.

In summary, moronecidin is a novel amphipathic α-helical 22-amino acid AMP. It has a broad spectrum of activity against a diverse array of microbes. Further study of moronecidin may yield an understanding about the mechanisms of salt-resistance and sensitivity among AMPs. Its remarkable salt resistance may make it useful for therapeutic applications in marine and human medicine. It may also serve as a template for designing new antibiotics with these particular characteristics.

Acknowledgments—We are grateful to Jean Paul Briand (CNRS Immunologie et Chimie Thérapeutiques, IBMC, UPR 9021, Strasbourg, France) for white bass moronecidin synthesis and to Patricia A. Jennings (University of California, Department of Chemistry and Biochemistry, La Jolla, CA) for analysis of circular dichroism spectroscopy. DNA sequencing was performed by the Molecular Pathology Shared Resource, University of California San Diego Cancer Center.

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