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The Chemotactic Response of *Vibrio anguillarum* to Fish Intestinal Mucus Is Mediated by a Combination of Multiple Mucus Components

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Chemotactic motility has previously been shown to be essential for the virulence of *Vibrio anguillarum* in waterborne infections of fish. To investigate the mechanisms by which chemotaxis may function during infection, mucus was isolated from the intestinal and skin epithelial surfaces of rainbow trout. Chemotaxis assays revealed that *V. anguillarum* swims towards both types of mucus, with a higher chemotactic response being observed for intestinal mucus. Work was performed to examine the basis, in terms of mucus composition, of this chemotactic response. Intestinal mucus was analyzed by using chromatographic and mass spectrometric techniques, and the compounds identified were tested in a chemotaxis assay to determine the attractants present. A number of mucus-associated components, in particular, amino acids and carbohydrates, acted as chemoattractants for *V. anguillarum*. Importantly, only upon combination of these attractants into a single mixture were levels of chemotactic activity similar to those of intestinal mucus generated. A comparative analysis of skin mucus revealed its free amino acid and carbohydrate content to be considerably lower than that of the more chemotactically active intestinal mucus. To study whether host specificity exists in relation to vibrio chemotaxis towards mucus, comparisons with a human *Vibrio* pathogen were made. A *cheR* mutant of a *Vibrio cholerae* El Tor strain was constructed, and it was found that *V. cholerae* and *V. anguillarum* exhibit a chemotactic response to mucus from several animal sources in addition to that from the human jejunum and fish epithelium, respectively.

Vibrio anguillarum is an important pathogen of marine fish species, being the major causative agent of a terminal hemorrhagic septicemia known as vibriosis (9, 28). In intensive aquaculture, outbreaks of vibriosis can severely deplete fish stocks (2) and hence, much effort is being directed towards understanding the events behind the pathogenic process of vibriosis. The modes of transmission of *Vibrio* fish pathogens have been determined to be waterborne (23) and foodborne (48) infection. A number of factors have been implicated in the virulence of *V. anguillarum*, including the iron-sequestering system (6, 7, 50), hemolytic and proteolytic extracellular products (22, 35, 55), lipopolysaccharide (38), and serum resistance (56). Like other members of the *Vibrio* genus, *V. anguillarum* exhibits rapid swimming motility in an aqueous milieu which is conferred by a polar flagellum. Previously, our laboratory revealed that chemotactic motility mediated by the polar flagellum is essential for virulence when fish are exposed to the pathogen by immersion in bacteria-containing water but not by intraperitoneal injection (42). It was subsequently considered important to elucidate possible mechanisms by which chemotactic motility is involved in the virulence of *V. anguillarum*.

The virulence findings imply that *V. anguillarum* responds chemotactically to certain fish-derived products in a manner that promotes the infection process prior to penetration of the fish epithelium. Different lines of evidence indicate that *V. anguillarum* can invade fish epithelium at more than one site, including the skin and the intestinal tract (10, 54). The skin is directly exposed to water containing the pathogen, and it has been shown that *V. anguillarum* adheres to skin mucus (4, 27) and can invade through experimentally created lesions on the skin (54), which suggests that this is a plausible route of infection in the case of injured fish. Furthermore, marine teleosts, in contrast to their freshwater counterparts, are known to continuously drink water (11), which would hence subject the gastrointestinal tract to waterborne infection. It has been demonstrated that orally ingested *V. anguillarum* can survive passage through the stomach of feeding fish (41) and that the intestinal tract is a site of adhesion (20, 40), colonization, and proliferation (41) for *V. anguillarum* whereby it can utilize intestinal mucus as a nutrient source (15, 39). In addition, oral or rectal administration of *V. anguillarum* to fish results in a systemic infection (17, 40) in which *V. anguillarum* is transported across the intestinal epithelium by endocytosis (17). Given that the fish skin and intestinal epithelial surfaces are protected by a layer of mucus, to invade the epithelium, disseminate within the host, and manifest vibriosis, *V. anguillarum* must first negotiate its way through the mucus barrier. To achieve such a feat, it became apparent that *V. anguillarum* may direct its passage towards and through mucus by using chemotactic motility whereby components of the mucus act as chemoattractants.

The primary objective of this study was to measure the chemotactic response of *V. anguillarum* to mucus from a natural host of vibriosis and to investigate the basis of any response with respect to mucus composition. The response of *V. anguillarum* wild type and a nonchemotactic mutant to mucus from rainbow trout was quantified in a chemotaxis assay. Biochemical analysis was performed on intestinal mucus to determine the nature of the chemoattractant(s) present, and comparative studies with skin mucus were made. We also examined

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whether another *Vibrio* pathogen, *Vibrio cholerae*, exhibited a chemotactic propensity specifically towards mucus from its preferred site of colonization, the human jejunum, in contrast to mucus from other animal sources. An open reading frame corresponding to the *cheR* homologue of *V. cholerae* was cloned and mutated to aid this investigation.

MATERIALS AND METHODS

Bacterial strains and plasmids. *V. anguillarum* NB10 (serotype O1) was isolated at the Umeå Marine Research Center, Norrbyn, Sweden, by our laboratory during a natural outbreak of vibriosis (37). *V. anguillarum* nonchemotactic mutant OTR27 was derived from strain NB10 following construction of a 411-bp in-frame deletion in the coding region of the *cheR* gene (42). OTR27 was complemented with wild-type *cheR* by homologous recombination of the suicide vector pNQ705.1 (31) containing the wild-type *cheR* gene of *V. anguillarum* (plasmid pCheR-Va) into the truncated *cheR* gene of OTR27. The resulting strain, OTR27/pCheR-Va, regained chemotactic motility in liquid broth and soft agar. *V. cholerae* CVD110 Δ(*ctxAB zot ace*) *hlyA*::(*ctxB mer*) Hg^r (32) is an attenuated derivative of O1 El Tor strain E7946 (29). *Escherichia coli* DH5α (Pharmacia) was used as a host strain for cloning experiments with pBluescript KS(+) (Stratagene). For the cloning of DNA fragments into pNQ705.1 (31), ligation products were transformed into the highly competent *E. coli* SY327 (34). pNQ705.1 recombinants to be conjugated into *Vibrio* strains were then transformed into *E. coli* S17-1 (49), which was used as the donor strain.

Media and growth conditions. *E. coli* was routinely grown at 37°C with Luria broth or agar (Bacto Laboratories). *V. anguillarum* and *V. cholerae* were grown at room temperature and at 37°C, respectively, in Trypticase soy broth (TSB) (BBL) or on TSB plus 1.5% agar. The soft agar used for assaying the motility of *Vibrio* strains was TSB plus 0.3% agar. The *Vibrio*-selective medium used was TCBS agar (Difco Laboratories). Antibiotic concentrations for *E. coli* strains were 100 μg of ampicillin/ml and 25 μg of chloramphenicol/ml. For *V. anguillarum* and *V. cholerae*, 10-μg/ml chloramphenicol was used.

Chemicals. Types II (crude) and III (partially purified) porcine gastric mucin, porcine and bovine bile, and all compounds tested in the chemotaxis assay or used for analytical purposes were purchased from Sigma Chemical Co. In all experiments, glass-distilled deionized water was used. Solvents used for organic extraction of mucus and in thin-layer chromatography (TLC) were of analytical grade. Solvents used for other chromatographic analyses and mass spectrometry (MS) were of high-performance liquid chromatography (HPLC) grade.

PCR, DNA sequencing, and enzymes. Oligonucleotide primers were synthesized with an Applied Biosystems model 394 automated DNA-RNA synthesizer (Perkin-Elmer). Unless stated otherwise, PCR cycle times and gel electrophoretic analysis of the products were as previously described (42). For DNA sequencing, the Applied Biosystems Prism dye terminator cycle sequencing kit and sequencer model 377 (Perkin-Elmer) were used. *Taq* DNA polymerase and restriction enzymes were purchased from Boehringer Mannheim. T4 DNA ligase was purchased from Promega. KGB buffer (46) was used for all restriction digests.

Isolation of rainbow trout mucus and bile. Rainbow trout (*Oncorhynchus mykiss*) weighing between 200 and 300 g were heavily anesthetized with tricaine methane sulfonate (Sigma Chemical Co.). The surface of each fish was rinsed with water, and the skin mucus was collected with a plastic spatula. The mucus from up to 12 fish was combined and stored at −20°C. For the collection of intestinal mucus, fish that had been kept in 10°C water without food for at least 4 weeks were used to ensure that virtually all of the food present in the gastrointestinal tract had been processed. The peritoneum of each fish was cut open sufficiently to expose the gastrointestinal tract. The intestine from the pylorus to the vent was removed and its outer surface was carefully cleaned of its layers of fat. Pressure was applied to the sides of the intestine so that the mucus exuded out through one of the open ends. The mucus was collected in sterile 1.5-ml polypropylene tubes, and any samples which contained traces of blood were discarded. The intestinal mucus from up to 12 fish (2 to 4 ml in total) was combined and stored at −20°C. For the chemotaxis assays, the crude skin and intestinal mucus gel was diluted 1/10 with water and homogenized by using a Potter-Elvehjem homogenizer and vortex shaker. Bile was obtained by inserting a fine sterile needle attached to a 0.5-ml syringe into the gall bladders of dissected fish and by slowly drawing out the bile, which was then stored at −20°C. Human intestinal mucus was a generous gift from Silvia Melgar (Department of Immunology, Umeå University) and was obtained as M199 tissue culture medium (plus dithiothreitol) washings of biopsy samples of human jejunum that had been rinsed of fecal material.

Chemotaxis assay. A modification of the quantitative capillary assay (1) was used to measure *Vibrio* chemotaxis. Strains of *V. anguillarum* and *V. cholerae* were grown in TSB overnight. In the case of *V. cholerae*, the overnight culture was diluted 10-fold in TSB and reincubated for up to 4 h to maximize the number of motile cells before proceeding to the assay. The bacteria were harvested by centrifugation at 6,000 × *g* for 5 min and resuspended in an equal volume of sterile 0.9% NaCl. This washing step was repeated three times, and the final resuspension was made in 1× chemotaxis (CTA) buffer, i.e., 10 mM sodium phosphate buffer (pH 7.0), to give an estimated cell density of 10^{10} bacteria/ml. Serial 10-fold dilutions of the bacterial suspension were made in 1× CTA buffer, and viable cell plating was performed. A suspension of bacteria in 1× CTA buffer at an estimated concentration of 10^7 viable bacteria/ml was dispensed in 200-μl aliquots into 1.5-ml polypropylene tubes. A 1-μl capillary tube (Drummond Scientific Co.), heat sealed at one end and containing the substrate (in 1× CTA buffer) to be tested in half the length of the tube, was inserted horizontally into the polypropylene tubes lying on their sides to approximately 0.5 cm below the surface of the bacterial solution. After incubating for 60 min at room temperature, the capillaries were removed and externally rinsed with water, and their contents were expelled into 300 μl of phosphate-buffered saline. Counts of viable cells were performed on the capillary contents. In each experiment, substrates were simultaneously tested in duplicate, and control capillaries containing 1× CTA buffer were included. The chemotactic activity of a particular substrate was expressed in terms of the relative response (RR), i.e., the ratio of mean accumulation of bacteria in substrate-containing capillaries to the mean accumulation of bacteria in the control capillaries. For direct comparison of bacterial accumulation in terms of cell numbers in assays where more than one bacterial strain was tested, slight deviations in the concentration of the bacterial suspension from the predicted concentration of 10^7 viable cells/ml were adjusted for with regard to each strain. Rainbow trout mucus was tested as a homogenate of crude mucus gel diluted 1/10. Commercial preparations of individual compounds were tested at the concentrations 0.1 and 1 mM and also at 10 mM for amino acids and carbohydrates. Commercial preparations of mucin and bile were tested in the range of 0.1 to 10 mg/ml.

Fractionation methods. The intestinal mucus homogenate (crude mucus gel diluted 1/10 corresponding to a dry weight of 15 mg/ml) was extracted with 2.5 volumes of chloroform-methanol (2:1 [vol/vol]). The mix was extensively shaken overnight by using a Griffin flask shaker and then allowed to separate into an aqueous and an organic phase. The aqueous phase was reextracted by using the organic phase from a chloroform-methanol-water (10:5:2 [vol/vol/vol]) system in which the subsequent extraction times were reduced to 1 h. In total, four extractions were made. The organic phases were combined, thus producing two fractions, an aqueous fraction (LE-Aq) and an organic fraction (LE-Org). For chemical analyses, the material was kept in methanol.

Analytical methods. (i) TLC. For TLC analyses, plates coated with silica gel 60 (Merck & Co., Inc.) were used. Samples were evaluated by developing in chloroform-methanol-water (75:25:3 [vol/vol/vol]). To visualize the material, different spraying reagents were used, since the sensitivity of nonspecific reagents, such as iodine vapor, was too low. Most lipids could be seen after spraying with *n*-(1-naphthyl)-ethylenediamine dihydrochloride (NEA) (5) and heating for 10 min at 110°C. To locate phospholipids and long-chain hydrocarbons, the plates were sprayed with the phosphate-group-specific molybdenum blue reagent (60) and left at room temperature. Using this procedure, phospholipids appeared as blue spots after 5 min, and neutral lipids appeared as white spots on a grey background after approximately 15 min. To screen for amino acids, fractions were developed in *n*-butanol–acetic acid–water (100:7:5 [vol/vol/vol]) and visualized using ninhydrine. Amino acids could be preliminarily identified and quantified by comparing them with amino acid standards of known concentrations.

(ii) GC and GC-MS. A 5972 MSD gas chromatography-MS (GC-MS) system (Hewlett-Packard) was used for the identification of compounds present in the extracts. Quantification of identified compounds was performed with a 5880 GC system (Hewlett-Packard) using flame ionization detection. Both instruments were equipped with a 7673 autosampler and a DB5 column (30-m length, 0.25-mm internal diameter, 0.25-μm film thickness; J&W Scientific) with helium as the carrier gas. Samples were introduced by splitless injection. After 1 min at 50°C, the column temperature was increased at a rate of 25°C/min to 170°C followed by 5°C/min to 280°C. The final temperature was held for 10 min. The injector temperature was 200°C and the detector-interface temperature was 280°C. The MS was operated in the electron ionization mode, and the acquired mass spectra were matched to those in the NBS-Wiley database integrated with the MS software.

Free fatty acids (FA) were identified and quantified in the mucus organic extract as their corresponding methyl ester derivatives (33). Cholesterol and mono- and diglycerides could be identified and quantified without derivatization. Carbohydrates were analyzed directly in the crude mucus gel as peracetylated alditol derivatives (47). To determine the presence of both free and bound carbohydrates, analysis was performed before and after hydrolysis of the mucus in 4 M trifluoroacetic acid at 100°C for 4 h.

Amino acids were analyzed in the crude mucus gel after derivatization with isobutyl chloroformate (57). As above, qualitative and quantitative analyses were performed using the 5972 MSD GC-MS and 5880 GC systems (Hewlett-Packard), respectively. A spectrum database was created by analyzing derivatized amino acid standards. The samples were introduced by splitless injection into a DB1701 column (30-m length, 0.25-mm internal diameter, 0.25-μm film thickness; J&W Scientific). The temperature was increased from 50°C to 190°C at a rate of 30°C/min and then to 280°C at a rate of 10°C/min. The final temperature was held for 10 min.

(iii) Liquid chromatography electrospray MS (LC-MS). The mucus organic extract was separated by HPLC using a reversed-phase Grom-Sil ODS4-HE column (10-cm length, 1.0-mm internal diameter; Grom Analytic). A Rheos 4000 HPLC pump (Crelab Instruments, AB, Karlskoga, Sweden) was used at a flow rate of 400 μl/min. A flow splitter, installed before the sample injection valve

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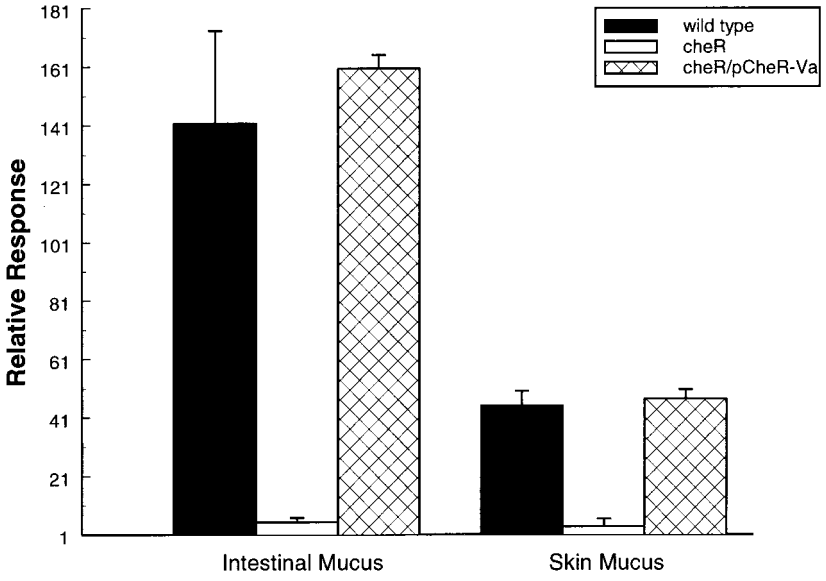

FIG. 1. Chemotactic response of *V. anguillarum* to rainbow trout intestinal and skin mucus. Chemotaxis was measured in a capillary assay for *V. anguillarum* NB10 (wild type), OTR27 (cheR), and complemented strain OTR27/pCheR-Va (cheR/pCheR-Va) and was expressed in terms of a relative response (the ratio of bacterial accumulation in substrate-containing capillaries to that in control buffer-containing capillaries). Mucus substrates consisted of a homogenized 1/10 dilution of crude mucus gel. Substrates contained 1× CTA buffer and were tested in duplicate. The accumulation of wild-type, *cheR* mutant, and the complemented strain in control buffer-containing capillaries was 102 ± 16.5, 90 ± 15, and 99 ± 10.5 viable bacteria, respectively, which correspond to an RR of 1 for each strain. Error bars represent average deviations.

(Valco Instruments Co.), reduced the flow rate to 30 μl/min. Mobile phase A was methanol-water (4:1 [vol/vol]), 5 mM ammonium acetate, and mobile phase B was methanol-chloroform (1:1 [vol/vol]), 5 mM ammonium acetate. A linear gradient from 100% A to 100% B over 10 min was applied 1 min after injection, followed by 5 min at 100% B. Bile acids were analyzed by using a gradient from acetonitrile-water (1:1 [vol/vol]) to 100% acetonitrile containing 0.05% trifluoroacetic acid. The separated material was introduced via a fused silica transfer line (75-μm internal diameter) to the electrospray source. An Autospec orthogonal acceleration–time-of-flight (TOF) unit (Micromass Ltd.) was used and the instrument was operated at an acceleration voltage of +4 kV and −4 kV in the positive and negative ion modes, respectively. The magnetic sector was bypassed, and the TOF analyzer was used to acquire mass spectra over the range of 1 to 2,000 at a resolution of 1,000. For analysis in the positive and negative ion modes, 10 mM ammonium acetate and 0.1% ammonium hydroxide, respectively, were added to the solutions. The sampling cone voltage was set at 50 V as a compromise between the optimal values for different compounds. Cone voltage fragmentation (CVF), i.e., fragmentation of compounds in the ion source, was induced by increasing the sampling cone voltage to 150 V.

For liquid chromatography tandem MS (LC-MS-MS) analysis, the parent ion was isolated by the magnetic sector analyzer and admitted to the collision cell. Methane was used as the collision target at a pressure of 1.5×10^{-4} Pa and a collision energy of 400 eV. Product ion spectra were acquired by the TOF analyzer by summing spectra during a 2-s interval. In the negative ion mode, the FA composition of phospholipid species could be established from the abundant carboxylate anions produced as previously described (26).

Construction of a nonchemotactic mutant of *V. cholerae*. A large set of primers used previously for the sequencing and manipulation of the *cheR* gene of *V. anguillarum* (42) were paired in numerous combinations. PCR was then performed on *V. cholerae* CVD110 and *V. anguillarum* NB10 (as a control) by using the collection of convergent primers and the following PCR cycle times: 94°C for 30 s, 50°C for 45 s, and 72°C for 45 s. Primer pairs producing single PCR products from *V. cholerae* CVD110 which correlated closely in size with the corresponding fragments obtained for *V. anguillarum* NB10 were retested under the same conditions. One PCR fragment, cheRF2R2, generated from CVD110 template was identical in size to the corresponding cheRF2R2 fragment from *V. anguillarum* which encompasses nearly the entire *cheR* coding region. The cheRF2R2 fragment obtained from CVD110 was cloned into the *Sac*I and *Xba*I restriction sites of pBluescript, and both strands were sequenced to determine whether it aligned with the *cheR* gene of *V. anguillarum* at the nucleotide and deduced amino acid levels.

Another PCR fragment generated from CVD110 template, cheRF3R3, which was similar in size to the corresponding internal cheRF3R3 fragment from the *V. anguillarum cheR* gene, was cloned into the *Cla*I and *Xba*I restriction endonuclease sites of the suicide vector pNQ705-1. The resulting recombinant plasmid, pRON131, was sequenced with respect to its insert to verify its identity to internal sequence of cheRF2R2 from *V. cholerae*. pRON131 was mobilized by conjugal mating into *V. cholerae* CVD110 as previously described (43), and conjugants were selected on TCBS agar containing chloramphenicol. Insertion of this plasmid by homologous recombination in the putative *cheR* gene of CVD110 was verified by PCR using a primer complementary to the plasmid just outside the linker region of pNQ705-1 and another primer complementary to the *V. cholerae cheR* sequence outside the cheRF3R3 region. The resulting *V. cholerae* strain, OTL131, was tested for chemotactic motility in liquid broth and soft agar. The two primers which generated fragment cheRF2R2 in the PCR were cheRF2 (5′-CTAGGAGCTCGCTATAACTATAAGCGATCAA) and cheRR2 (5′-CTAGTCTAGAGTTTATAAATGATGCCAGGG). The two PCR primers which generated fragment cheRF3R3 were cheRF3 (5′-CTAGATCGATTCTTCAGGTCAAGAGCCTTAC-3′) and cheRR3 (5′-CTAGTCTAGAACGTTACGACAGAAAATAAT-3′). Database searches were conducted by using the sequence analysis software of the Genetics Computer Group, University of Wisconsin (8), and other programs available from the National Center for Biotechnology Information (35a).

Nucleotide sequence accession number. The *V. cholerae* DNA sequence present on fragment cheRF2R2 described above represents a partial sequence of the putative *cheR* gene of *V. cholerae* and has been deposited into GenBank under the accession no. AF139167.

RESULTS

Chemotactic response of *V. anguillarum* to fish mucus. A large accumulation (RR, 142) of wild-type *V. anguillarum* cells in capillaries containing the intestinal mucus homogenate was observed with respect to the control capillaries containing CTA buffer (Fig. 1). A comparatively weaker chemotactic response by wild-type *V. anguillarum* towards skin mucus was detected (RR, 45; Fig. 1). The motile nonchemotactic *cheR* mutant OTR27 was isolated from the mucus-containing capillaries in dramatically reduced numbers, while complementation of OTR27 with the *cheR* gene restored the wild-type chemotactic phenotype (Fig. 1).

Analysis of the intestinal mucus material. To examine the content of intestinal mucus, the material was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), TLC, GC, GC-MS, and LC-MS. The compounds identified are listed in Tables 1 and 2 at their respective con-

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TABLE 1. Chemotactic response of *V. anguillarum* to amino acids and carbohydrates identified and quantified in rainbow trout intestinal mucus[a]

Amino acid or carbohydrate	Relative response[b]	Concentration (mM) in intestinal mucus[c]
Amino acids		
L-Alanine	<3	2.53
L-Asparagine	<3	0.73
L-Aspartic acid	<3	1.05
L-Glutamic acid	6	3.03
L-Glutamine	49	2.02
Glycine	24	3.16
L-Histidine	45	0.97
L-Isoleucine	42	1.09
L-Leucine	7	2.0
L-Lysine	<3	1.74
L-Phenylalanine	<3	0.99
L-Proline	<3	1.51
L-Serine	37	1.57
L-Threonine	51	1.33
L-Tryptophan	<3	0.2
L-Tyrosine	<3	1.67
L-Valine	<3	1.69
Carbohydrates		
L-Fucose	6	0.20[d]
D-Galactose	<3	0.17[d]
D-Glucose	12	0.07
myo-Inositol	<3	0.12
D-Mannose	14	0.05
N-acetyl-D-galactosamine	<3	0.08
N-acetyl-D-glucosamine	<3	0.11
D-Ribose	<3	0.46
2-Deoxy-D-ribose	<3	0.26
D-Xylose	9	0.13

[a] The chemotactic response of wild-type *V. anguillarum* NB10 to individual mucus-associated amino acids and carbohydrates at 0.1, 1.0, and 10 mM concentrations was measured in a capillary assay and is expressed in terms of a relative response (the ratio of bacterial accumulation in substrate-containing capillaries to that in control buffer-containing capillaries).
[b] Relative response for each substrate when present at a concentration of 1.0 mM is illustrated.
[c] The concentration of the individual components in undiluted crude intestinal mucus gel.
[d] Concentration was determined following hydrolysis of the sample.

centrations in the crude mucus gel. Slight variations in the concentrations of individual compounds in different mucus batches were observed, and the quantities listed are average values obtained from a number of mucus samples.

(i) Protein, amino acid, and carbohydrate analyses. The main determinants of mucus structure are heavily glycosylated proteins known as mucins (12). The presence of glycosylated proteins in fish intestinal mucus was confirmed by periodic acid-Schiff staining (53) of SDS-PAGE-separated proteins electroblotted onto polyvinylidene difluoride membranes (data not shown). In TLC analysis, free amino acids were visualized by using ninhydrine (Fig. 2A). GC and GC-MS determined the identities and approximate concentrations of the amino acids in the crude mucus gel. Seventeen of the 20 common amino acids were detected in the mucus (Table 1). (Arginine, cysteine, and methionine were not detected.)

GC-MS analysis was also used to identify carbohydrates in crude mucus. Before hydrolysis of the sample, *N*-acetylgalactosamine, *N*-acetylglucosamine, 2-deoxyribose, glucose, mannose, ribose, xylose, and inositol appeared as free carbohydrates (Table 1). Furthermore, following hydrolysis, fucose, galactose, and increased amounts of *N*-acetylgatactosamine and *N*-acetylglucosamine were detected.

TABLE 2. Chemotactic response of *V. anguillarum* to lipids identified and quantified in rainbow trout intestinal mucus[a]

Lipid group[d]	Relative response[b]	Concentration (μg/ml) in intestinal mucus[c]
Free FAs (16:0, 18:0, 18:1, 20:1, 20:4, 20:5, 22:6)	<3	500
Mono- and diglycerides (MPG, MSG, DSG)	<3	200
Cholesterol	<3	150
Phospholipids (PC, PI)	<3	100
Bile acids		
TC	5	16
TCDOC	5	4

[a] The chemotactic response of wild-type *V. anguillarum* NB10 to individual mucus-associated lipids at 0.1 and 1.0 mM concentrations was measured in a capillary assay and expressed in terms of a relative response (the ratio of bacterial accumulation in substrate-containing capillaries to that in control buffer-containing capillaries).
[b] Relative response for each substrate when present at a concentration of 1.0 mM is illustrated.
[c] The concentration of the individual components in undiluted intestinal mucus gel derived from their respective measured concentrations in a chloroform-methanol extract of the 1/10-diluted intestinal mucus homogenate.
[d] MPG, monopalmitoylglycerol; MSG, monostearoylglycerol; DSG, distearoylglycerol; PC, phosphatidylcholine; PI, phosphatidylinositol.

(ii) Lipid analysis. In addition to mucins, mucus is known to contain various types of lipids (51, 52, 58). To analyze the lipid content, the intestinal mucus homogenate was extracted with chloroform-methanol, producing aqueous (LE-Aq) and organic (LE-Org) fractions. An initial examination of the composition of the extracts was obtained by TLC. The staining

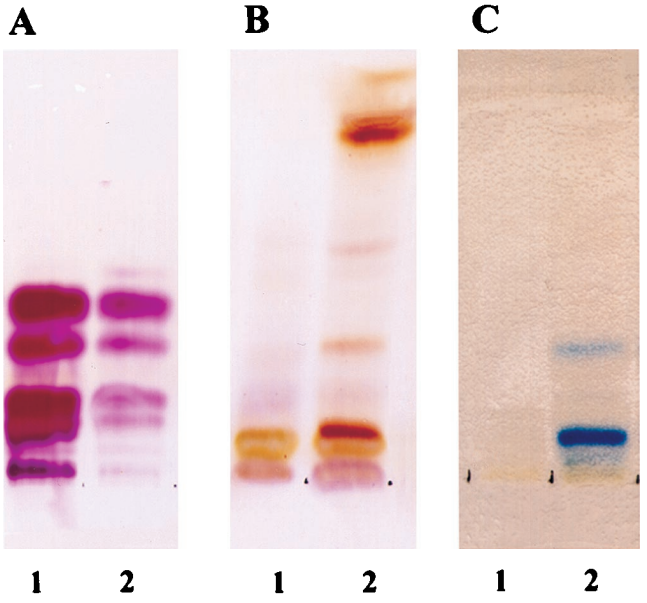

FIG. 2. TLC analysis of the fish intestinal mucus fractions obtained after organic solvent extraction. Initial compositional analysis of the rainbow trout intestinal mucus was performed by TLC. Fractions LE-Aq (aqueous) and LE-Org (organic) were obtained after chloroform-methanol extraction of intestinal mucus homogenate. The mucus fractions were developed in chloroform-methanol-water (75:25:3 [vol/vol/vol]). (A) Ninhydrine staining to visualize amino acids. (B) *n*-(1-naphthyl)-ethylenediamine dihydrochloride (NEA) staining to visualize lipids. (C) Molybdenum blue reagent staining to visualize phospholipids. Lanes: 1, LE-Aq; 2, LE-Org.

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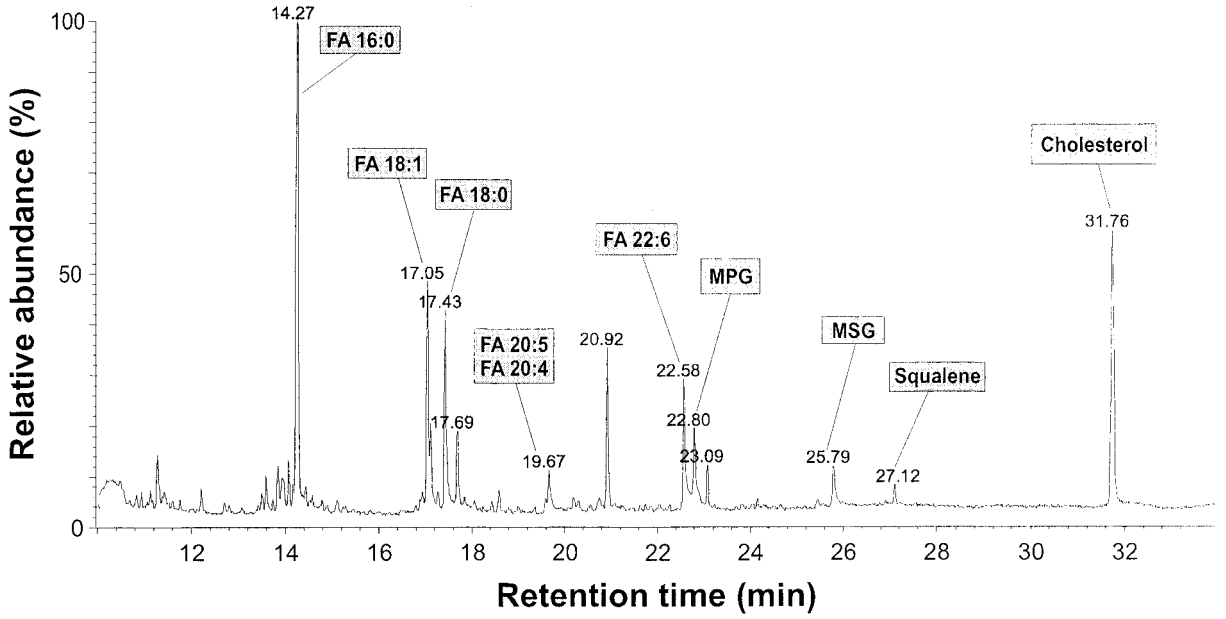

FIG. 3. GC-MS analysis of the LE-Org fish intestinal mucus fraction. LE-Org was analyzed following esterification and methylation. Identification was achieved by comparison of the GC retention times with those of known standards and by matching the mass spectra from MS analysis to the NBS-Wiley database. Cholesterol, FAs, monoacylglycerides, and squalene were detected by this procedure. The signals appearing at 17.69 and 20.92 min correspond to amide forms of FAs. MPG, monopalmitoylglycerol; MSG, monostearoylglycerol.

reagent NEA revealed that most of the mucus-associated lipids localized to LE-Org (Fig. 2B). The use of appropriate standards indicated that the upper doublet seen in the chromatogram of LE-Org (Fig. 2B) consists of cholesterol (upper band) and polyunsaturated FAs (lower band). Saturated FAs did not stain with NEA but could be seen following rhodamine staining (data not shown). Phospholipids were specifically detected in the lower part of the chromatogram by using the phosphate-group-specific molybdenum blue reagent (Fig. 2C). Using phospholipid standards, a phospholipid which shared mobility properties with phophatidylcholine was revealed (data not shown).

Analysis by GC-MS confirmed the presence of cholesterol and free FAs in LE-Org and also revealed the presence of monoglycerides (Fig. 3) and their approximate concentrations were determined by using standards (Table 2). The diglyceride distearoylglycerol was also detected in LE-Org. As with TLC analysis, a significant amount of unsaturated FAs was found in LE-Org, which is consistent with a previous report concerning fish lipid extracts (44). Among these, arachidonic acid (FA 20:4), eicosapentaenoic (FA 20:5), and docosahexaenoic acid (FA 22:6) were found. Other FAs detected consisted of palmitic acid (FA 16:0), stearic acid (FA 18:0), and oleic acid (FA 18:1).

LC-MS and LC-MS-MS analysis in both the positive (ES+) and negative (ES−) ion modes were used to further characterize the LE-Org extract (Fig. 4A). Free FAs and phospholipids were identified and in addition a group of polar lipids, which were not detectable using GC-MS, were determined by LC-MS to be bile acids. Under reversed-phase conditions, the bile acids and FAs eluted early in the chromatogram (Fig. 4A). Phospholipids constituted the middle part of the chromatogram, while glycerides eluted last (Fig. 4A).

By comparing their retention times with those of known standards, the bile acids present were identified as taurocholic acid (TC) and taurochenodeoxycholic acid (TCDOC) (Fig. 4B). Quantification of the bile acids was performed by LC-MS using the $(M-H)^-$, i.e., deprotonated molecular ion, at mass-to-charge ratios (m/z) of 514 and 498 for TC and TCDOC, respectively. It was found that TC and TCDOC accounted for approximately 80 and 20%, respectively, of the bile present. For comparison purposes, bile isolated directly from the gall bladder of rainbow trout was analyzed. As with LE-Org, TC and TCDOC were the predominant cholic acid types and were also present at a ratio of approximately 4:1 (data not shown). The identities of the FAs, already established by the GC-MS analysis, were confirmed by LC-MS (Fig. 4C). In addition, eicossenoic acid (FA 20:1) was detected by LC-MS.

The presence of phospholipids was also determined by LC-MS analysis. In the positive ion mode under CVF conditions, choline glycerophospholipids were localized in the chromatogram by a characteristic fragment ion at an m/z of 184 originating from the choline moiety (data not shown). The phospholipids were further characterized in separate LC-MS-MS experiments using ES− by selection of the molecular ion, $(M-H \text{ plus acetate})^-$ for choline glycerophospholipids and $(M-H)^-$ for inositol glycerophospholipids, and recording the product ion spectrum. The peak at 8.33 min (Fig. 4D) in the m/z 881 trace gave a product ion spectrum with fragment ions and mass losses characteristic for phosphatidylinositol substituted with FA 16:0/22:6 (data not shown). Similarly, for the m/z 818 trace, the peak at 10.50 min (Fig. 4D) was identified as phosphatidylcholine substituted with FA 16:0/18:1 (data not shown).

Chemotactic response towards individual mucus components. Commercial preparations of compounds identified in the mucus material were individually tested in the chemotaxis assay at the concentrations 0.1, 1.0, and 10 mM. The chemotactic responses to individual compounds at a concentration of 1 mM are illustrated in Tables 1 and 2. Compounds for which the relative response was consistently less than 3.0 were not considered significant chemoattractants. Of the compounds

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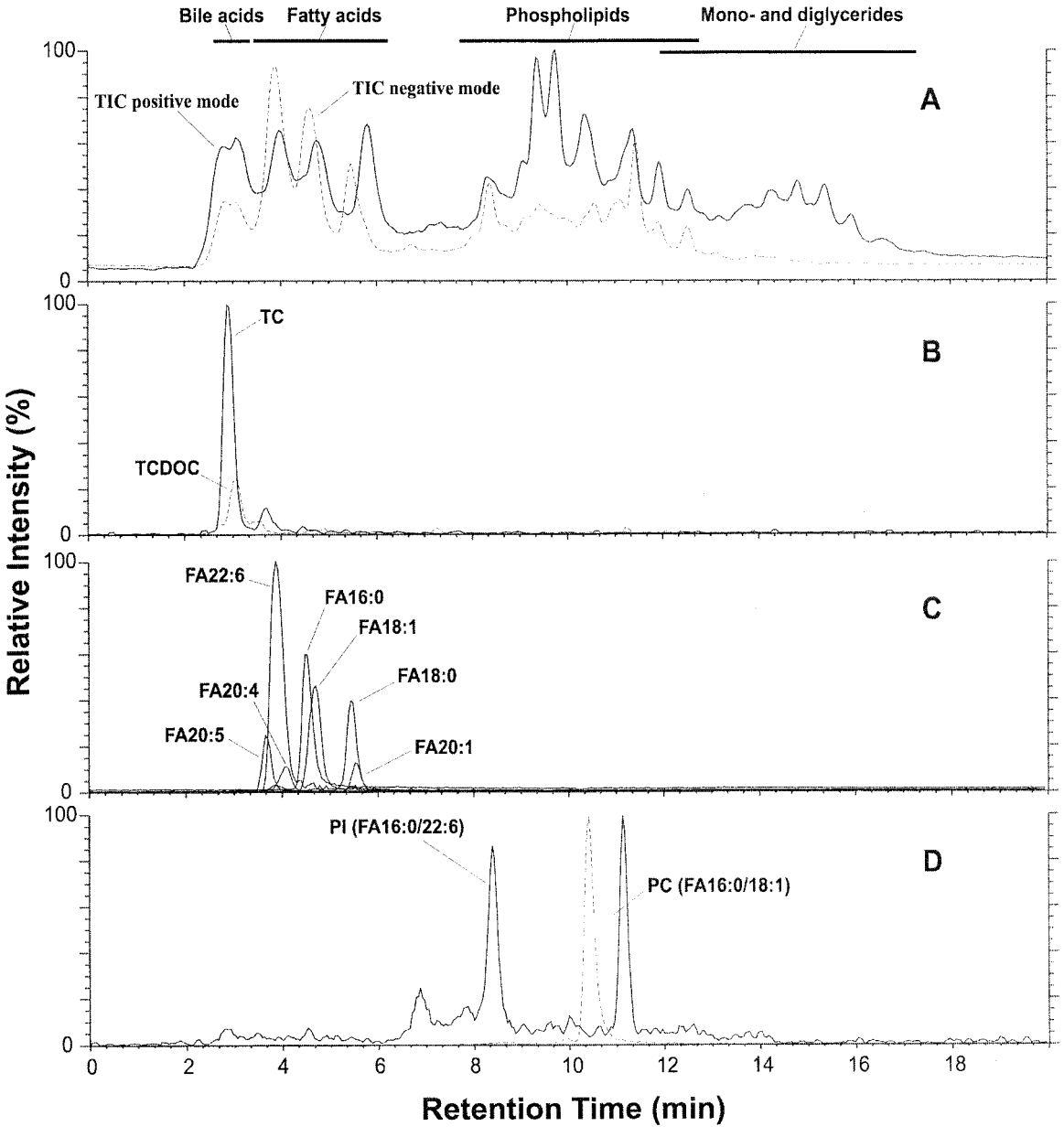

FIG. 4. LC-MS analysis of the LE-Org fish intestinal mucus fraction. LE-Org was separated on a reversed-phase HPLC column and analyzed by both positive and negative ion mode electrospray MS. The elution order of the compounds present is indicated above panel A. (A) TIC positive mode, the total ion current trace (TIC) obtained in the positive ion mode which represents mainly protonated $(M+H)^+$ and ammonium adduct $(M+NH_4)^+$ molecular ions; TIC negative mode, the TIC trace obtained in the negative ion mode which represents mainly deprotonated $(M-H)^-$ molecular ions. (B) Extracted ion chromatograms in the negative ion mode of *m/z* 514 and *m/z* 498, corresponding to $(M-H)^-$ of the bile acids TC and TCDOC, respectively. The chromatogram was normalized to the intensity of TC. (C) Extracted ion chromatograms in the negative ion mode of *m/z* corresponding to the indicated FAs. All chromatograms were normalized to the intensity of *m/z* 327, corresponding to FA 22:6. (D) PI, extracted ion chromatogram in the negative ion mode of *m/z* 881, corresponding to $(M-H)^-$ of phosphatidylinositol substituted with FA 16:0/22:6; PC, extracted ion chromatogram in the negative ion mode of *m/z* 818, corresponding to $(M-H + \text{acetate})^-$ of phosphatidylcholine substituted with FA 16:0/18:1.

identified in the intestinal mucus, the amino acids glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, serine, and threonine and the carbohydrates fucose, glucose, mannose, and xylose displayed relative responses above 3.0 and thus represented chemoattractants for *V. anguillarum* (Table 1). Two lipid compounds found in LE-Org, TC and TCDOC, also generated relative responses above 3.0 (Table 2). Commercial preparations of the individual components identified in the intestinal mucus were each combined at the following two concentrations: (i) their respective concentrations in the intestinal mucus homogenate, i.e., crude mucus gel diluted 1/10 and homogenized and (ii) 0.1 mM. All mucus-associated attractants and in addition, all mucus components with individual relative responses below 3.0 were combined at the above concentrations, and the resulting mixtures were tested in the chemotaxis assay to compare their activities with that of the intestinal mucus homogenate. Combination of each of the mucus attractants at a concentration of 0.1 mM produced a mixture

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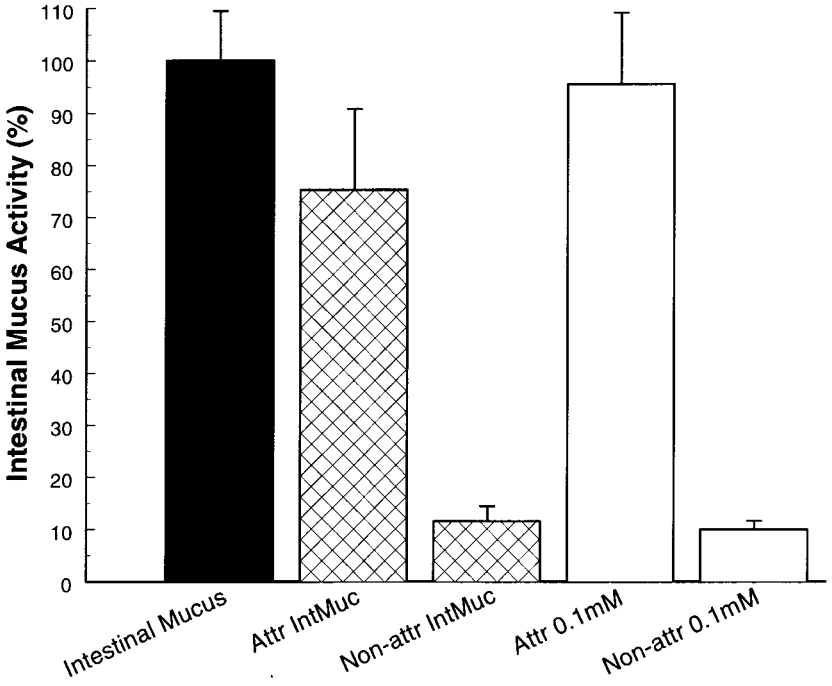

FIG. 5. Chemotactic activity of combinations of components identified in rainbow trout intestinal mucus. Chemotaxis was measured in a capillary assay for wild-type *V. anguillarum* NB10 and is expressed as a percentage of the chemotactic activity, i.e., RR, of the intestinal mucus homogenate (crude mucus gel diluted 1/10 and homogenized) which was set at 100%. Commercial preparations of the individual components identified in the intestinal mucus were each combined at the following two concentrations: (i) their respective concentrations in the intestinal mucus homogenate (IntMuc); and (ii) 0.1 mM. The above combinations were made with all mucus-associated attractants (Attr) and with all mucus compounds with individual relative responses below 3.0 (Non-Attr). The accumulation of wild-type bacteria in control buffer-containing capillaries was 75 ± 12 viable bacteria, which corresponds to a relative response value of 1. All substrates contained 1× CTA buffer and were tested in duplicate. Error bars represent average deviations.

with 95% of the activity of the intestinal mucus (Fig. 5). Similarly, combination of the attractants at their corresponding concentrations in the intestinal mucus homogenate produced a mixture with 75% of the chemotactic activity of intestinal mucus (Fig. 5). In contrast, mixtures containing mucus components with individual RRs below 3.0 possessed less than 12% of the activity of intestinal mucus (Fig. 5).

Analysis of the skin mucus material. The above results demonstrate that intestinal mucus contains a range of chemoattractants, the majority of these consisting of free amino acids and carbohydrates. To enable comparisons between mucus from the intestinal and skin epithelia, the skin mucus was similarly analyzed with respect to its amino acid and carbohydrate composition. This analysis revealed that the concentration of free amino acids and carbohydrates in skin mucus is considerably lower than that in intestinal mucus. Skin mucus has a content of chemoattractant amino acids (i.e., glutamic acid, [1.5 mM], glycine, [0.4 mM], histidine, [0.06 mM], and leucine, [0.15 mM]; glutamine, isoleucine, serine, and threonine were not detected) and carbohydrates (only fucose [0.076 mM] and mannose [0.05 mM] were detected before sample hydrolysis) lower than that of intestinal mucus (Table 1).

Cloning and mutagenesis of the putative *cheR* open reading frame of *V. cholerae*. PCR using the *V. anguillarum cheR* primers cheRF2 and cheRR2 produced an approximately 810-bp fragment when both *V. cholerae* CVD110 and *V. anguillarum* NB10 cells were used as template. The 773 bp of *V. cholerae* DNA sequence (i.e., between and excluding the complementary sites of the *V. anguillarum* primers) over its entire length exhibited 77.5 and 92.2% identity at the nucleotide and deduced amino acid levels, respectively, to the *cheR* gene of *V. anguillarum* and, furthermore, showed considerable homology to known *cheR* genes from other bacterial species. The 773 bp of *V. cholerae* sequence represents a partial coding sequence and encompasses nearly the entire *cheR* gene with respect to *cheR* of *V. anguillarum* (828 bp; GenBank accession no. U36378).

PCR using the *V. anguillarum* internal *cheR* primers cheRF3 and cheRR3 produced an approximately 360-bp fragment when both *V. cholerae* CVD110 and *V. anguillarum* NB10 cells were used as template. To generate a *V. cholerae cheR* mutant, the cheRF3R3 PCR fragment from CVD110 template was cloned into the suicide vector pNQ705-1, generating plasmid pRON131. Sequencing confirmed that cheRF3R3 exactly matched an internal region of the putative *V. cholerae cheR* sequence present on fragment cheRF2R2. pRON131 was mobilized into *V. cholerae* CVD110 by conjugal mating, and PCR analysis verified that the plasmid had integrated into the *cheR* gene homologue of CVD110. The resulting mutant, OTL131, exhibited rapid nonchemotactic motility in liquid broth and failed to swarm through soft agar, in contrast to parent strain CVD110, which confirmed the involvement of this *V. cholerae cheR* gene homologue in chemotaxis.

Chemotactic response of *V. cholerae* to mucus and bile from various sources. The chemotactic response of wild-type and *cheR* mutant strains of *V. cholerae* towards mucus and bile from a number of sources was assayed. Porcine gastric mucus and porcine and bovine bile extracts induced an accumulation of wild-type *V. cholerae* in the capillaries (Fig. 6). In addition, a strong chemotactic response by wild-type *V. cholerae* towards human jejunal mucus (RR, 253) with respect to the M199 media control was observed (Fig. 6). *V. cholerae* also exhibited

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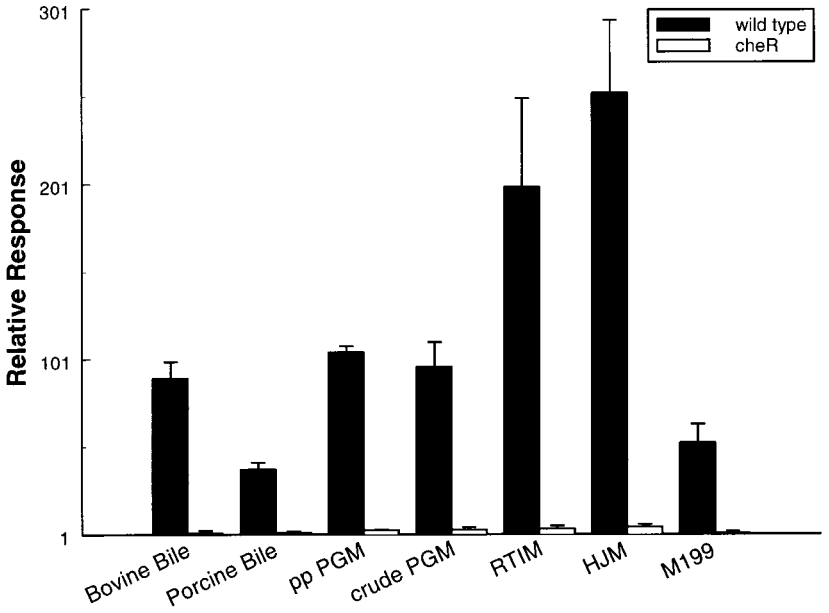

FIG. 6. Chemotactic response of *V. cholerae* to mucus and bile from various sources. Chemotaxis was measured in a capillary assay for *V. cholerae* CVD110 (wild type) and OTL131 (*cheR* mutant) and expressed in terms of an RR (the ratio of bacterial accumulation in substrate-containing capillaries to that in control buffer-containing capillaries). pp PGM, 10-mg/ml commercial partially purified porcine gastric mucin; crude PGM, 10-mg/ml commercial crude porcine gastric mucin; RTIM, rainbow trout intestinal mucus homogenate; HJM, crude human jejunal mucus from M199 (tissue culture media) washings of human jejunum biopsy samples. Control capillaries containing M199 media were also included in the assay. Bovine bile and porcine bile represent 10-mg/ml concentrations of respective commercial preparations. All substrates contained 1× CTA buffer and were tested in duplicate. The accumulations of wild-type and *cheR* mutant in control buffer-containing capillaries were 76.5 ± 16.5 and 87 ± 7.5 viable bacteria, respectively, which corresponds to an RR value of 1 for each strain. Error bars represent average deviations.

chemotaxis towards rainbow trout intestinal mucus (RR, 199; Fig. 6). *V. anguillarum* was similarly attracted to the above mucus and bile substrates (data not shown).

DISCUSSION

In this study, the response of *V. anguillarum* towards skin and intestinal mucus from rainbow trout was measured in a chemotaxis capillary assay. It was found that wild-type *V. anguillarum* manifested a strong chemotactic response towards intestinal mucus which was abolished in the *cheR* mutant (Fig. 1). The response by *V. anguillarum* to skin mucus, which was also dependent upon a fully intact chemotaxis machinery, was relatively lower (Fig. 1). Work was then performed to identify the chemoattractants present in fish mucus. Given the increasing evidence which implicates the fish intestinal tract as being a site of epithelial invasion by *V. anguillarum* (17, 40) and the high chemotactic activity of its mucus layer (Fig. 1), intestinal mucus was initially chosen for further analysis. The mucus material was examined with respect to its protein, amino acid, carbohydrate and lipid content using a variety of techniques including SDS-PAGE, TLC, GC-MS, and LC-MS.

As expected, large glycosylated proteins, mucins, were associated with the mucus (data not shown). Furthermore, free amino acids (Fig. 2A) and carbohydrates were detected in the crude mucus material, while hydrolysis of the mucus material liberated the monosaccharides fucose and galactose and increased amounts of *N*-acetylgalactosamine and *N*-acetylglucosamine (Table 1). This indicates that the latter carbohydrates may be present as mucin-bound moieties in fish intestinal mucus as is the case for mucus from other animal species (45).

For the analysis of lipids, the mucus was extracted with chloroform-methanol, and the resulting organic (LE-Org) and aqueous (LE-Aq) fractions were examined. The majority of the mucus-associated lipids partitioned to LE-Org (Fig. 2B and C), which was then analyzed and found to contain saturated and unsaturated free FAs, phospholipids, bile acids, cholesterol, and mono- and diglycerides (Fig. 3 and 4). The dominant phospholipids present in mucus were identified as phophatidylcholine and phosphatidylinositol (Fig. 4D). The bile acids present consisted of TC and TCDOC (Fig. 4B), which is consistent with the types of cholic acids identified in bile isolated from the gall bladder of rainbow trout in this work and in a previous study by other researchers (16). Bile is a regular constituent of the intestinal tract (19) and is known to contain cholesterol and phospholipids, in particular, phosphatidylcholine (18). Thus, in addition to the bile acids, a number of other lipid components of fish intestinal mucus may also have originated from the gall bladder.

Commercial preparations of the compounds identified in the mucus material were tested individually for chemotactic activity with respect to *V. anguillarum*. Of the free amino acids identified in the intestinal mucus, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, serine, and threonine behaved as chemoattractants (Table 1). Furthermore, the carbohydrates fucose, glucose, mannose, and xylose were chemotactic (Table 1). Of the lipid compounds identified, the bile acids TC and TCDOC induced a weak chemotactic response (Table 2). Bile isolated directly from rainbow trout also possessed chemotactic activity for *V. anguillarum* (data not shown).

By combining all individual chemoattractants identified into a single mixture, it was possible to reconstitute a high level of chemotactic activity similar to that present in the intestinal mucus homogenate (Fig. 5). In contrast, when presented alone, none of the mucus components possessed chemotactic activity equivalent to that of intestinal mucus. Thus, rather than con-

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taining a single potent chemoattractant, it appears that intestinal mucus consists of a range of chemoattractants which contribute to the overall activity of the sample.

The bulk of the chemoattractants identified in intestinal mucus consist of free amino acids and carbohydrates. The less-active skin mucus was therefore analyzed with respect to its amino acid and carbohydrate contents to permit comparisons between mucus from the intestinal and skin epithelia. The concentrations of free amino acids and carbohydrates, including those that act as chemoattractants, were found to be considerably lower in skin mucus than in intestinal mucus. This would provide a possible explanation for the lower chemotactic activity observed for skin mucus.

Regarding the role of chemotaxis in the virulence of *V. anguillarum*, deductions can be made from what is known about *V. cholerae* and other human enteropathogens, such as *Campylobacter jejuni*. Upon ingestion, *V. cholerae* colonizes the small intestine of humans from which it mounts its pathogenic effects on the host (3). *V. cholerae* exerts a chemotactic response towards the mucus layer which has been correlated with the pathogen's competence in penetrating the mucus and reaching the deep intervillous spaces (13, 14). This would facilitate the close association between the pathogen and the mucosal surface via pili which is considered important for both colonization of the intestine (24) and the efficient delivery of secreted toxins to epithelial cells (30). Thus, the decreased movement (compared to that of the wild type) of the *cheR* mutant of *V. anguillarum* towards fish mucus in vitro is likely to be mirrored by an impaired ability to penetrate the epithelial mucus and come in contact with and invade the epithelial surface during infection. Such reasoning would account for the attenuated virulence seen for the *cheR* mutant when presented to fish in the surrounding water (42). The ability of *C. jejuni* to localize itself in the mucus lining of the intestinal tract has also been associated with this pathogen's requirement for chemotaxis in host colonization and virulence (59).

A number of the fish intestinal mucus chemoattractants identified in this study are also present as bacterial chemoattractants in the mucus of other host species. For example, serine and fucose are mucus-associated attractants for the human pathogens *C. jejuni* (21) and *Pseudomonas aeruginosa* (36) and the porcine-infecting *Serpulina hyodysenteriae* (25). This would imply that the bacterial attraction to fish mucus may not be confined to fish pathogens such as *V. anguillarum* and alternatively, that fish pathogens may respond to mucus from other animal hosts. To investigate this, the partial coding region of the *cheR* gene of a *V. cholerae* El Tor strain was cloned and mutated. Chemotaxis assays demonstrated that *V. cholerae* requires a functional chemotactic system for movement towards mucus from its preferred site of colonization, the human jejunum (Fig. 6). Furthermore, *V. cholerae* displayed chemotaxis to mucus from other animal sources, namely, the porcine stomach and rainbow trout intestine (Fig. 6). Likewise, *V. anguillarum* exhibited a chemotactic response to human and porcine mucus (data not shown). It is therefore apparent that host specificity does not exist in relation to the chemotactic response of pathogenic vibrios to mucus. In addition, some variation in the content of the rainbow trout mucus batches was observed in this work, and it is likely that similar variations also occur in mucus from one fish species to another. However, the ability of *V. anguillarum* to respond to a range of mucus chemoattractants, as opposed to being dependent on the presence of one major chemoattractant, may be beneficial to the pathogen. This would enable *V. anguillarum* to respond to and penetrate mucus despite variations in composition and thus the pathogen would not be restricted in its capacity to infect different fish epithelia and, indeed, different fish species, on the basis of mucus content. Similarly, other mucophilic bacterial pathogens may overcome mucus variations in different individuals susceptible to infection by exploiting a range of chemotactic mucus constituents.

In conclusion, we have illustrated that chemotactic motility mediates movement of the pathogens *V. anguillarum* and *V. cholerae* towards mucus from their respective hosts and, in addition, to mucus from other animal sources. Analyses of fish intestinal mucus allowed the identification of several mucus-associated components consisting of amino acids, carbohydrates, and bile acids as chemoattractants for *V. anguillarum*. Combination of the individual mucus attractants generated mixtures with levels of chemotactic activity similar to that exhibited by intestinal mucus. It is proposed that the presence of multiple chemoattractants in host mucus has implications for the relationship between chemotaxis and bacterial virulence.

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