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Identification and characterization of peptidases secreted by quahog parasite unknown (QPX), the protistan parasite of hard clams

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ABSTRACT: Quahog parasite unknown (QPX) is a protistan parasite capable of causing deadly infections in the hard clam *Mercenaria mercenaria*, one of the most valuable shellfish species in the USA. QPX is an extracellular parasite found mostly in the connective tissue of clam mantle and, in more severe cases of infection, other clam organs. Histopathologic examinations revealed that QPX cells within clam tissues are typically surrounded by hollow areas that have been hypothesized to be, at least in part, a result of extracellular digestion of clam proteins by the parasite. We investigated peptidase activity in QPX extracellular secretions using sodium dodecyl sulfate-polyacrylamide gels containing gelatin as a co-polymerized substrate. Multiple peptidase activity bands of molecular weights ranging from 20 to 100 kDa were detected in QPX secretions derived from a variety of culture media. One major band of approximately 35 kDa was composed of subtilisin-like peptidases that were released by QPX cells in all studied media, suggesting that these are the most common peptidases used by QPX for nutrient acquisition. PCR quantification of mRNA encoding QPX subtilisins revealed that their expression changes with the protein substrate used in the culture media. A fast protein liquid chromatography (FPLC) was used to fractionate QPX extracellular secretions. An FPLC-fraction containing a subtilisin-type serine peptidase was able to digest clam plasma proteins, suggesting that this peptidase might be involved in the disease process, and making it a good candidate for further investigation as a possible virulence factor of the parasite.

KEY WORDS: Quahog parasite unknown · Virulence factors · Peptidases · Zymography

INTRODUCTION

Peptidases or proteases are enzymes that hydrolyze proteins. In pathogenic organisms peptidases serve as virulence factors, enabling or facilitating infection processes (McKerrow et al. 1993, 2006, Klemba & Goldberg 2002). Specifically, secreted peptidases digest host proteins providing nutrients and allowing for infection spread, thus playing a key role in pathogenicity of various microbial agents including bacteria (Miyoshi & Shinoda 2000), fungi (Monod et al. 2002), and protozoa (Carruthers 2006, Alvarez et al. 2012). For example, peptidases of pathogenic organisms break down the components of the host extracellular matrix, such as collagen or fibrin, and components of blood and the immune system, such as hemoglobin, proteins of the complement system, and immunoglobulins (McKerrow et al. 1993, Klemba & Goldberg 2002, Lecaille et al. 2002).

Quahog parasite unknown (QPX) is a protistan parasite capable of causing deadly infections in the hard clam *Mercenaria mercenaria*, one of the most valu-

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able shellfish species in the USA. QPX infection outbreaks occur in wild and aquacultured hard clam stocks along the north-eastern coast of the USA and maritime Canada, leading to significant economic losses to the industry in those regions. QPX belongs to the thraustochytrids, a group of protists mostly consisting of osmo-heterotrophic and saprophytic organisms (Raghukumar 2002). It has been speculated that QPX mainly uses extracellular digestion to acquire nutrients, and possibly uses secreted peptidases to digest clam tissues during infection (Anderson et al. 2006). The QPX transcriptome contains over 200 sequences encoding for a variety of peptidases of all major types: serine (SPs), cysteine (CPs), aspartate (APs), threonine (TPs), and metallopeptidases (MPs) (Rubin et al. 2014). Over 70 of these peptidases possess an N-terminal signal peptide, suggesting that they are potentially directed into the secretory pathway (Rubin et al. 2015). In addition, 8 of these peptidases were detected in QPX extracellular products (ECPs) using liquid chromatography coupled with mass spectrometry (LC/MS) (Rubin et al. 2015).

The aim of this study was to investigate the types of peptidases secreted by QPX cells cultivated under various culture conditions and among 3 QPX isolates from different geographic locations. We hypothesized that QPX peptidase secretion will be variable and dependent on the protein supplement added to the growth medium. We also hypothesized that different QPX isolates will show similar peptidase secretion profiles. In addition, mRNA levels of candidate extracellular peptidases were measured to determine if transcriptional changes of peptidase expression also occur in response to culture conditions. Ultrafiltration and ion exchange chromatography were used to purify a major QPX peptidase before assessing its activity using clam plasma substrate polyacrylamide gels.

MATERIALS AND METHODS

In silico identification of QPX extracellular peptidases

The identification of peptidases predicted to be secreted by QPX cells was accomplished as described previously (Rubin et al. 2015). Briefly, translated open reading frames (ORFs) from 2 QPX transcriptome libraries (11 005 and 12 579 ORFs) were screened for the presence of an N-terminal signal peptide, which is known to direct the protein into the secretory pathway (Petersen et al. 2011). In total, 1255 unique proteins were predicted to constitute the QPX secretome, and these included 74 putative extracellular peptidases (Rubin et al. 2015). In the current study, the translated amino acid sequences of these 74 peptidases were further grouped into peptidase families based on classification by the peptidase database, MEROPS (Rawlings et al. 2012). Amino acid sequence alignments of peptidases belonging to the same family were generated using the ClustalW algorithm plug-in within Geneious v. 7.1.4 software (www.geneious.com; Kearse et al. 2012). Percent amino acid sequence identity between peptidases belonging to the same families and their expected molecular weights (MWs) were calculated using the same software.

QPX cultures and media

To compare transcriptional expression of QPX peptidases in response to different protein supplements, the following 4 media were prepared: minimal essential medium supplemented with fetal bovine serum (MEM FBS; Kleinschuster et al. 1998), MEM supplemented with yeastolate (MEM YSTO), MEM supplemented with gelatin (MEM GEL), and MEM supplemented with clam adductor muscle homogenate (MEM CAMH; Perrigault et al. 2009). The protein concentrations in all protein supplements were estimated using the Pierce BCA Protein Assay Kit (Thermo Scientific) and adjusted to a concentration of 2 mg ml^{-1} with sterilized artificial seawater (SASW; Instant Ocean®Sea Salt, 28 ppt). For the experimental cultures, 1 volume of MEM was mixed with 1 volume of 1 of the 4 protein supplements, resulting in a final protein concentration of 1 mg ml^{-1}. The cultures were prepared in 12-well plates filled with 4 ml of media in triplicate for each treatment. The QPX cell inoculum (NY1, isolate NY0313808BC7; Qian et al. 2007) was prepared from an exponentially growing culture in MEM and FBS. The cells were collected by centrifugation (3000 × *g*, 15 min at room temperature), washed twice with SASW, and re-suspended in un-supplemented MEM. Cell concentration of QPX inocula was determined with a hemocytometer, and the starting concentration of QPX was 8×10^3 cells ml^{-1} in all cultures. QPX growth was monitored by cell counts using a hemocytometer every other day, and QPX cells were collected when they reached similar cell concentrations: MEM FBS (2.3×10^6 cells ml^{-1}), MEM GEL (2.1×10^6 cells ml^{-1}), MEM YSTO (2.0×10^6 cells ml^{-1}), and MEM CAMH (1.7×10^6 cells ml^{-1}). To collect the cells

and QPX secretions (media supernatant), 1 volume of QPX culture was diluted with an equal volume of SASW and passed several times through a syringe to facilitate liquefaction of QPX extracellular mucus. The mixtures were transferred into 15 ml conical tubes and centrifuged at 3000 × *g* (20 min at 20°C). The supernatant was collected into 1.5 ml tubes as 40 µl aliquots. To each aliquot, 10 µl of 5× Laemmli loading dye, containing SDS but no beta-mercaptoethanol, were added and the aliquots were stored in a −80°C freezer until zymogram analyses. The cells were used for RNA extractions (see below), which were conducted on the day of collection. As the zymogram analysis of QPX secretions from the above described cultures revealed peptidase activities only for MEM FBS medium (see results below, Fig. 2A), a new culture based on MEM with 10% (v/v) of FBS (about 4 mg protein ml^{-1}) was used to check for peptidase activity. Since preliminary investigations showed that increased protein concentrations improve the sensitivity of the zymograms, additional cultures of the same QPX isolate were prepared using MEM supplemented with FBS and CAMH, both adjusted to 3 mg protein ml^{-1}. A lower concentration was selected (3 instead of 4 mg ml^{-1}) because of technical limitations for preparing CAMH stock solution at much higher protein concentration. QPX cultures were incubated for 7 d at 20°C before the supernatant was collected and preserved as described above. The QPX cell concentrations at the time of collection were 5.9×10^6 cells ml^{-1} and 6.4×10^6 cells ml^{-1} for FBS and CAMH, respectively.

For comparison of peptidase activities among different QPX isolates, 3 NY isolates were cultivated in MEM supplemented with 10% (v/v) of gelatin hydrolysate, yeastolate, and peptone solution (MEM GYP). Each supplement was prepared in seawater at 10 mg ml^{-1} (w/v) and sterilized by autoclaving. This media formulation was used because it was shown to be optimal for extracellular peptidase production for zymogram analysis in preliminary experiments. Two different QPX isolates originating from Raritan Bay, NY (NY0313808BC7 and NY0314220AC6, here designated NY1 and NY2, respectively) and 1 QPX isolate (NY070348D, or NY3) originating from a different NY embayment (Peconic Bay) were selected for peptidase expression comparison. QPX cultures (in triplicate) were established in 25 ml canted neck culture flasks (Falcon) with 7 ml of media and 0.3 ml of parasite cells suspended in SASW at 8.6×10^5 cells ml^{-1}. All cultures were incubated at 20°C for 11 d when the cell concentration reached 3.2×10^6 for NY1, 3.5×10^6 for NY2, and 1.4×10^6 for NY3 isolates (mean, n = 3). The cells and supernatants were collected as described above.

Zymography

SDS-PAGE was carried out in the presence of gelatin as a substrate copolymerized in the gels (zymography; Lantz & Ciborowski 1994) to detect proteolytic activity in QPX secretions. Gelatin type A from porcine skin (G9136, Sigma) was incorporated (0.1%) in either 10 or 12% polyacrylamide gels. Supernatant samples containing the loading dye (see previous section) were thawed but not boiled, to retain non-denatured structure of the peptidases. A 20 µl aliquot of sample was loaded into each lane. The gel electrophoresis was run at a constant 125 V in a standard Laemmli Tris-glycine SDS buffer. To remove the SDS, the gels were washed twice in 2.5% Triton-X100 in 0.1M Tris-HCl buffer (pH = 8.0) for 30 min on a rotating platform (80 rpm) at room temperature. To allow for enzyme digestion of gelatin, the gels were incubated in 0.1 M Tris-HCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$ buffer (pH = 8.0) at 37°C overnight. For inhibition assays, the gels were incubated overnight in the same 0.1 M Tris-HCl buffer (pH = 8.0) containing 1 of the following 8 inhibitors: EDTA (Fisher Scientific), trans-epoxy succinyl amido (4-guanidino) butane (E-64; Cat. no. E3132, Sigma), chymostatin (Cat. no. C7268, Sigma), phenylmethylsulfonyl fluoride (PMSF; Cat. no. P7626, Sigma), aprotinin (Cat. no. A1153, Sigma), tosylphenylalanylchloromethane (TPCK; Cat. no. T4376, Sigma), N_α-Tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK; Cat. no. T7254, Sigma), or antipain (Cat. no. 291907, Santa Cruz Biotechnology). All inhibitors were used at a final concentration equal to the maximal effective concentration suggested by the manufacturers: 10 mM PMSF, 1 mM EDTA, 10 µM E-64, 100 µM chymostatin, 0.8 µM aprotinin, 100 µM TPCK, 100 µM TLCK, and 100 µM antipain. After the incubation, the gels were stained for 10 to 15 min with a solution containing 0.23% (w/v) Coomassie blue, 5.8% (v/v) glacial acetic acid, and 30% methanol (v/v) in water and destained for 1 h in 10% acetic acid and 10% methanol.

Differential transcript expression of QPX peptidases

Five sequences coding for peptidases belonging to the subtilisin-like family (labeled S8-1 to S8-5) were

Table 1. Nucleotide sequences of PCR primers (F: forward; R: reverse) used to quantify mRNA levels of QPX peptidases

Gene ID	qPCR primer sequences			
S8-1	F:	TCGTGCTGGACACATAGTTGTCGT	R:	TATCGGTGGCTCCAACGCTTATCA
S8-2	F:	TATGGCTACTCCATTTGTCGCTGG	R:	ACGAGCAAATTGGGAGATTCGTGC
S8-3	F:	AAGAGCGGTTGGGAATATGGGAGT	R:	AACAACAAGCATGCCCTCTTCTGC
S8-4	F:	TTGCCGGTGTATTGGCTACGCTTT	R:	CTCGAGGTTTGCACCAACCAGTTT
S8-5	F:	TTGATGCAAGGCGTTTCCGATGTC	R:	GCGTCGATCGAAATGGCAAGTTGT
Actin	F:	TGAAGATCTTGACCGAGCGTGGTT	R:	AGCGGTCTTCATCTCCTGGTCAAA

selected to investigate their expression patterns in response to the 4 media and among the 3 QPX isolates. The subtilisins were selected as the zymography and inhibitor application indicated that subtilisin-like peptidases were the major QPX enzymes, and previous data showed that S8 peptidases are secreted by QPX cells (Rubin et al. 2015). The housekeeping gene beta-actin was used for mRNA normalization because its expression levels did not change with different growth conditions (Rubin et al. 2014). All primers were designed using the Primer 3 plug-in in Geneious software to amplify a PCR product between 100 and 200 bp in length (Table 1). For genes having similar nucleotide sequences (max identity 78% between S8-3 and S8-5), the final primer pairs were selected to be located in the least conserved regions of the nucleotide alignments.

Trizol reagent (Molecular Research Center) and the manufacturer's protocol were used to isolate RNA from all samples, and RNA quality and quantity were estimated spectrophotometrically by Nanodrop (Thermo Scientific). Total RNA (2.0 µg) from each sample was used to synthesize cDNA using the Moloney Murine Leukemia Virus Reverse Transcription kit (MMLV-RT, Promega) and 0.5 µg of oligo dT18 primers following the manufacturer's protocol. Relative quantification was carried out in 10 µl reactions with Brilliant II SYBR Green qPCR master mix (Agilent), 100 nM final primer concentration, and 5 ng of RNA-equivalent cDNA. The PCR reactions were performed using a Mastercycler EP Realplex PCR machine (Eppendorf). The peptidase expression levels were normalized to the beta-actin gene, and relative transcript levels were calculated using the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen 2001).

Purification of QPX subtilisin

The QPX isolate NY070348D (NY3) was cultivated in MEM supplemented with 10% (v/v) gelatin hydrolysate, yeastolate, and peptone solution (each at 10 mg ml^{-1} (w/v) in SASW. Six 70 ml flasks were filled with 15 ml of medium and inoculated with 1 ml of starting culture (8 d old QPX culture in the same medium). The cultures were incubated at 20°C for 10 d. For mucus collection, 10 ml of SASW was added to each 16 ml culture and mixed with a syringe to facilitate liquefaction of muco-polysaccharides. The total of 156 ml seawater and QPX culture mixture was distributed into 15 ml conical Falcon tubes and centrifuged for 15 min at 3000 × *g* at room temperature. The supernatant was collected into new 15 ml tubes and centrifuged for another 15 min to remove any remaining cells. Cell-free supernatant was passed through 15 ml 100 kDa ultrafiltration devices (Amicon) to remove QPX muco-polysaccharides, and the flow-through was concentrated using 3 kDa ultrafiltration devices (Amicon). The concentrated sample of 2 ml volume was dialyzed 3 times against 20 mM Tris HCl buffer at pH = 8.0. The sample was then fractionated by anion exchange fast-flow chromatography using an FPLC (AKTA system, GE Life Sciences). The sample was loaded onto a Q-sepharose fast-flow column (Q-FF 1 ml, GE Life Sciences), previously equilibrated with 20 mM Tris-HCl, pH 8.0 (buffer A). After washing the column with buffer A, the elution was carried out with an increasing gradient of 1 M NaCl mixed with Tris-HCl buffer (buffer B). Stock buffer B contained 20 mM Tris-HCl (pH 8.0), 1 M NaCl. After sample injection, a gradient was applied as follows: 20% buffer B over 15 min, 45% buffer B over 40 min, and a final step to 100% buffer B for 10 min. FPLC fractions identified by protein spectrophotometric signals were separated using SDS-PAGE, and peptidase activity of each fraction was revealed on gelatin and clam plasma substrate zymograms. The clam plasma substrate gels were prepared by substituting water in the gel mix with pooled clam plasma (33% volume of gel mix), which was withdrawn with a needle and a syringe from adductor muscles of 3 different clams.

Table 2. QPX peptidases (n = 74) containing an N-terminal secretion peptide sequence, grouped into families based on classification by the MEROPS peptidase database; members of families shaded in grey were previously detected in QPX secretions by LC/MS. Molecular weight (MW; kDa) calculated using Geneious v. 7.1.4; values in parentheses represent the number of peptidases with the preceding MW. na: complete open reading frame not available

MEROPS family	Family name	n	Calculated MW (kDa)	Inhibitor(s)
Serine				
S1	Trypsins/chymotrypsins	6	29, 30, 36, 41, 50, 74	Aprotinin, TLCK, PMSF
S8	Subtilases/subtilisins	7	39–43 (6), 77	DFP, PMSF
S10	Carboxypeptidases	11	41, 49–52 (7), 55, 62, 67	Chymostatin, antipain, PMSF, DFP
S28	Pro-Xaa carboxypeptidase	3	52, 54, 75	Lys thiazolidide
S54	Rhamboid peptidases	1	31	TPCK and 3,4-DCI
Cysteine				
C1	Papain-like peptidases	18	na, 22, 35, 36, 39, 45, 47, 48, 49, 52, 53 (2), 56, 60, 61 (2), 70, 75	E-64, leupeptin
C13	Legumain-like peptidases	2	na, 49	Iodoacetamide, N-ethylmaleimide
C26	Gamma-glutamyl hydrolase	3	34, 37, 38	Azaserine, 6-diazo-5-oxo-L-norleucine
Metallo				
M6	Immune inhibitor A peptidase	1	83	1,10-phenanthroline, EDTA
M10	Metzincins	1	na	1,10-phenanthroline, EDTA
M12	Astacins/ADAMS/adamalysins	6	41, 47, 53, 57, 86, 100	1,10-phenanthroline, EDTA
M14	Carboxypeptidases A	3	44, 52, 83	1,10-phenanthroline, EDTA
M20	Aminohydrolases	1	55	1,10-phenanthroline, EDTA
M35	Deuterolysins	2	49, 56	1,10-phenanthroline, EDTA
Aspartic				
A1	Pepsins	6	46, 50, 53, 66, 97, 173	Pepstatin
A22B	Presenilin	1	42	Pepstatin
Threonine				
T1	Proteasome peptidases	2	26 (2)	No inhibitors

RESULTS

In silico identification of QPX extracellular peptidases

Over 200 transcripts coding for a variety of peptidases were identified in 2 different QPX transcriptome libraries (Rubin et al. 2015). Based on the classification of the MEROPS comprehensive peptidase database (Rawlings et al. 2012), QPX possesses intra- and extracellular peptidases belonging to 38 different peptidase families and all major catalytic types including metallo-, serine, cysteine, aspartate, and threonine peptidases. Further amino acid sequence analyses and alignments of QPX peptidase-coding transcripts revealed that 74 of these peptidases possess an N-terminal signal peptide for the secretory pathway (Table 2), and thus were predicted to be extracellular. These peptidases belong to 17 different families, with 3 families represented by the highest number of transcripts: C1, S8, and S10 (Table 2). Eight of the 74 predicted peptidases have been experimentally confirmed to be secreted by QPX cells cultivated in MEM supplemented with 0.3% (w/v) yeastolate (Rubin et al. 2015). These 8 extracellular peptidases include: 3 subtilisin-like peptidases (MEROPS family: S8), 2 papain-like peptidases (C1), 1 carboxypeptidase (S10), and 2 metallopeptidases (M35 and M12B) (Rubin et al. 2015, this study; marked in grey in Table 2).

Based on currently available QPX transcriptome libraries, the highest number of QPX putative extracellular peptidases (18 different transcripts; Table 2) belongs to the C1 family, also known as the papain-like peptidase family. The assignment of peptidase family is based on sequence similarity matches; however, not all QPX peptidases have the expected active residues. For example, peptidases in the C1 family contain 3 conserved amino acid residues known as the catalytic triad: cysteine, histidine, and asparagine (C-H-N) (Lecaille et al. 2002, Sajid & McKerrow 2002, Atkinson et al. 2009), in which the sulfhydryl group (-SH) of the cysteine residue is involved in the hydrolysis of the peptide bond (Sajid & McKerrow 2002). However, the 3 active residues were only detected in 14 out of the 18 C1 peptidase ORFs, suggesting that the remaining 4 might be non-peptidase homologs. Ten of the C1 peptidases of QPX were classified into the C1A subfamily (also known as cathepsins), which is characterized by 4 active residues with an additional glutamine preceding the active triad (Q-C-H-N; Fig. 1A) (Sajid & McKerrow 2002).

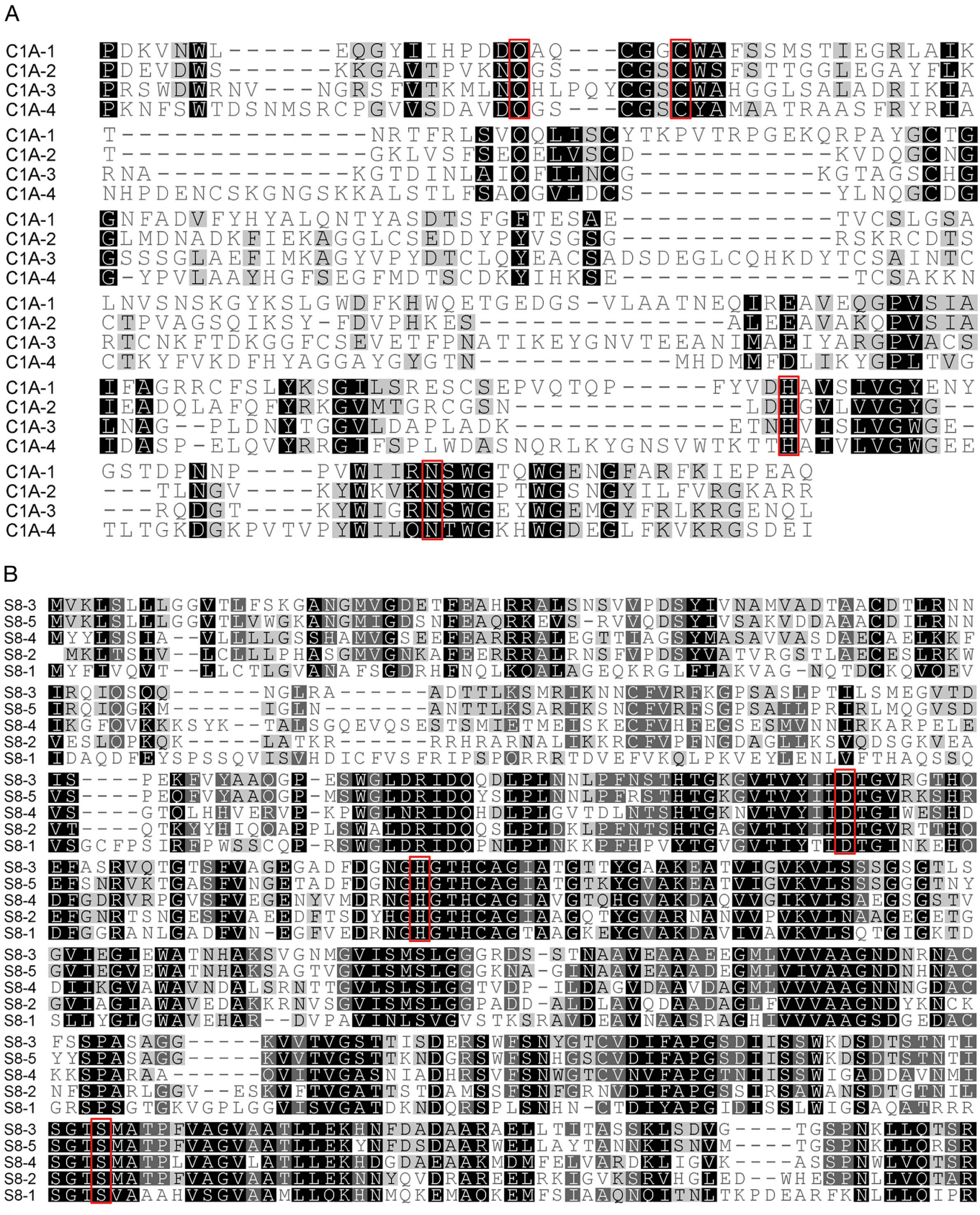

Fig. 1. Amino acid alignments of QPX peptidases: (A) papain-like family (C1) and (B) subtilisin-like family (S8). Red boxes mark the catalytic residues: Q-C-H-N for the C1A peptidase subfamily and D-H-S for the S8 family

Table 3. Summary of peptidase activity bands (—) detected in QPX secretions on multiple zymograms; QPX secretions were derived from different media for NY1 isolate and from 3 different QPX isolates (NY1, NY2, NY3) grown in the same media. MW: molecular weight; MEM: minimal essential medium; YSTO: yeastolate; FBS: fetal bovine serum; GEL: gelatin; CAMH: clam adductor muscle homogenate; GYP: mix of gelatin hydrolysate, yeastolate, and peptone. Protein concentration in each medium is shown in parentheses

MW (kDa)	MEM with YSTO (1 mg ml^{-1})	MEM with FBS (1 mg ml^{-1})	MEM with GEL (1 mg ml^{-1})	MEM with CAMH (1 mg ml^{-1})	CAMH (3 mg ml^{-1})	MEM FBS (3 mg ml^{-1})	MEM FBS (4 mg ml^{-1})	MEM GYP NY1 (3 mg ml^{-1})	MEM GYP NY2 (3 mg ml^{-1})	MEM GYP NY3 (3 mg ml^{-1})
20							—	—	—	—
30		—			—	—	—	—	—	—
40					—	—	—	—	—	
75						—			—	
90						—		—		
100						—				—

The second highest number of predicted extracellular peptidases belongs to the serine carboxypeptidase family (S10 family). QPX possesses 11 different S10 peptidases (Table 2) of predicted MW ranging from 41 to 67 kDa (Table 2). The third most numerous group of extracellular QPX peptidases is the S8 serine peptidase family. Peptidases of the S8 family have a catalytic triad in the order aspartic acid (D), histidine (H), and serine (S) (Fig. 1B), which is a different order to that of other serine peptidases such as the S10 family (alignment not shown). QPX possesses 7 subtilisin-like peptidases (S8) containing a signal peptide (Table 2), and the calculated MW of 6 S8 peptidases was between 39 and 43 kDa. One of the S8 peptidases has a C-terminal extension rich in proline and threonine residues, suggesting it is anchored to the cell surface or is glycosylated. The amino acid sequence alignment of 5 QPX subtilisins revealed between 36 and 79% pairwise nucleotide identity (data not shown).

Peptidase activity in QPX secretions

The supernatants from the experimental cultures were analyzed for peptidase activity by gelatin substrate SDS-PAGE. Different proteolytic activity bands were detected in the secretions of QPX cells grown in different media for 1 isolate (NY1) and among 3 different QPX isolates cultured in the same medium (Table 3). When QPX cells were cultivated in MEM supplemented with 1 of 4 different protein sources (gelatin, yeastolate, FBS, and CAMH) at the final protein concentration of 1 mg ml^{-1}, only 1 peptidase activity band (30 kDa) was revealed for only 1 medium, viz. MEM supplemented with FBS (Table 3, Fig. 2A). Two bands of peptidase activity (30 and 40 kDa) were detectable in QPX secretions derived from CAMH cultures (without MEM, 3 mg ml^{-1} protein concentration; Table 3, Fig. 2B). The maximum number of peptidase bands (6 in total) was revealed when QPX (NY1) was grown in MEM supplemented with FBS. However, different MW bands were revealed from 2 separate sets of cultures: subculture A, 5 peptidase bands from MEM FBS culture adjusted at 3 mg ml^{-1} of total protein concentration (Fig. 2B); and subculture B, 3 bands from MEM FBS culture at 4 mg ml^{-1} (Fig. 2C). Two of the bands (30 and 40 kDa, Table 3) were the same between the 2 culture conditions, but the high MW bands (>75 kDa; Table 3, Fig. 2B) were only visible in 1 of the QPX subcultures. It is possible that some of the lower MW bands are single peptide chains of the higher MW multi-chain peptidases. The actual source of perceived difference between the 2 QPX subcultures would have to be tested experimentally.

The most pronounced/thickest band in MEM FBS media was around 40 kDa MW and was partially inhibited by 2 serine peptidase inhibitors, viz. PMSF and chymostatin (Fig. 2C). This band was not inhibited by the cysteine peptidase inhibitor, E-64 (Fig. 2D), or the metallo-peptidase inhibitor, EDTA (data not shown), suggesting that it is primarily composed of serine peptidases. The band is most likely composed of multiple peptidases because it contains at least 1 peptidase resistant to PMSF and chymostatin inhibition (Fig. 2C). In addition, the band was not inhibited by any other tested serine peptidase inhibitor: TLCK, TPCK, aprotinin, and antipain (data not shown), suggesting that the band is mostly composed of subtilisin-type (S8 family) peptidases. The other 2 bands (20 and 30 kDa) that were present in QPX secretion from MEM FBS (at 4 mg ml^{-1} protein concentration) cultures were also inhibited by the

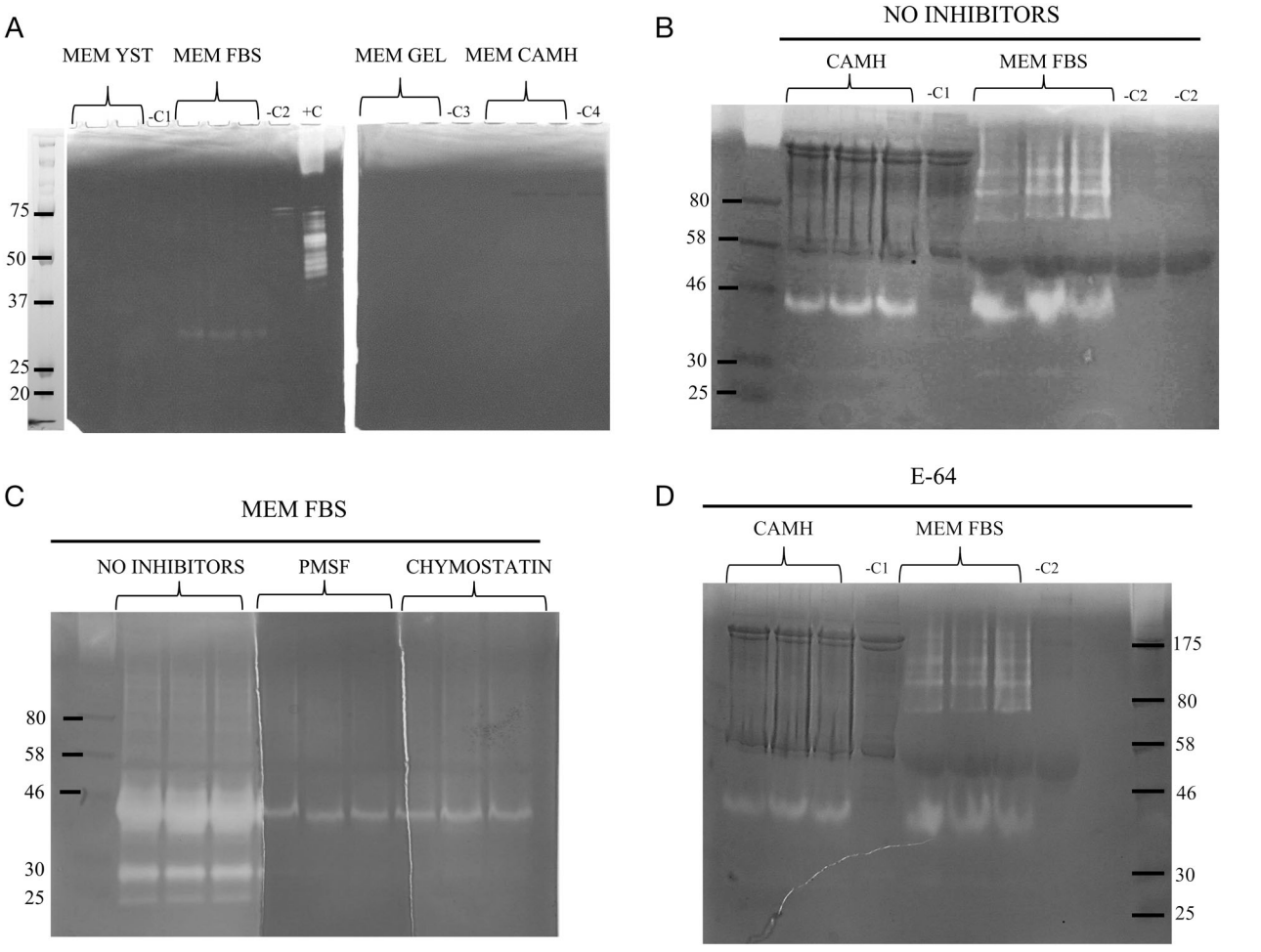

Fig. 2. Zymograms showing extracellular peptidases produced by QPX (NY1 isolate) grown: (A) in minimal essential medium (MEM) supplemented with either yeastolate (YSTO), fetal bovine serum (FBS), gelatin (GEL), or clam adductor muscle homogenate (CAMH), to a final protein concentration of 1 mg ml^{-1}; and (B) in MEM FBS and CAMH with protein concentration adjusted to 3 mg ml^{-1}. –C1 to –C4 are media incubated in parallel with the cultures but without QPX cells ; +C: positive control (30 ng of collagenase). (C) Effect of peptidase inhibitors (10 mM PMSF and 100 µM chymostatin) on extracellular peptidases produced by QPX cells grown in MEM FBS (total protein concentration ~4 mg ml^{-1}; in triplicate). (D) Effect of E-64 (cysteine peptidase inhibitor) at 10 µM final concentration on peptidases in QPX extracellular product samples from panel B

serine peptidase inhibitors PMSF and chymostatin (Fig. 2C). These 2 low MW peptidase bands were markedly stronger when the media were supplemented with higher concentration of FBS (3 and 4 mg ml^{-1}, Fig. 2B vs. 2C). The 2 peptidase bands of lowest MW were not observed when frozen supernatant was used, preventing their further identification using inhibitors.

When QPX cells were cultivated in MEM supplemented with gelatin, yeastolate, and peptone, each at the final concentration of 1 or 3 mg ml^{-1} total, the extracellular peptidase production was at its highest, indicated by the brightest activity bands on the zymograms (Fig. 3A). Three different QPX isolates were grown in this medium, and the ECPs derived from those cultures differed in the peptidase activity band pattern (Table 3, Fig. 3A). NY2 QPX had a peptidase band running at approximately 75 kDa, which was not present in ECPs from NY1 and NY3 isolates, but possibly corresponds to high MW bands of about 90 and 100 kDa that were present in the secretions of NY1 and NY3 isolates, respectively (Fig. 3B). All peptidases were partially degraded after freezing (Fig. 3A vs. 3B), and the 2 peptidase bands of MW just below 37 kDa (present for all 3 isolates) were inhibited by PMSF (Fig. 3B vs. 3C) but not by cysteine peptidase inhibitor (E-64) or metallopeptidase inhibitor (EDTA; results are exactly the same as Fig. 2D, i.e. non-inhibition by E-64 and EDTA was recorded, but not documented by photography).

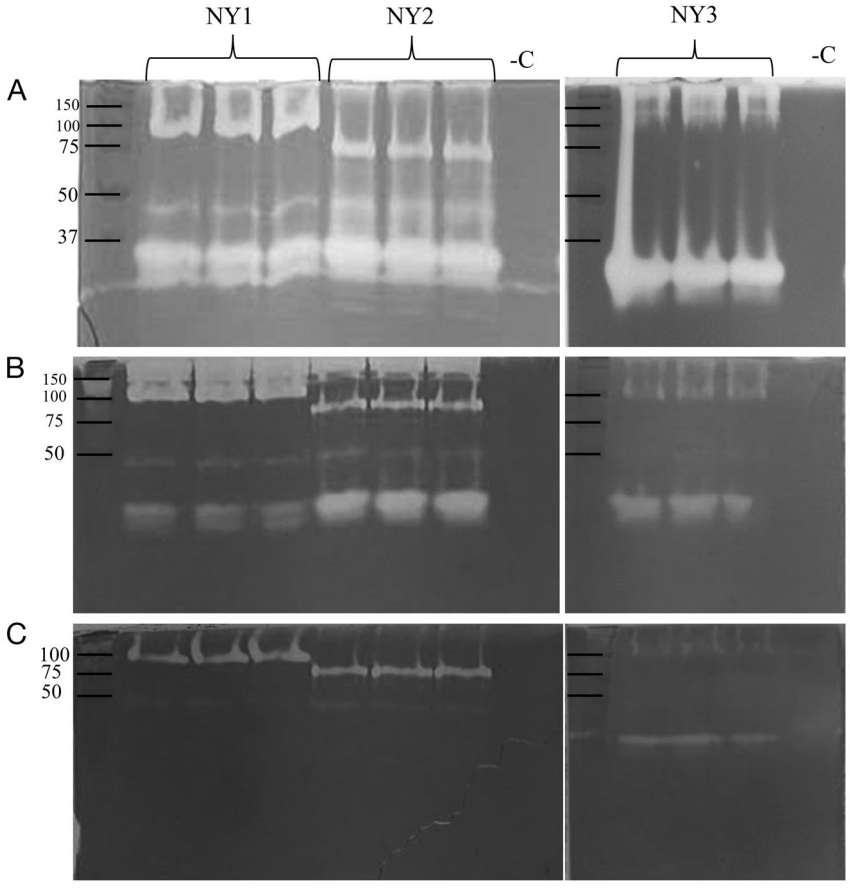

Fig. 3. Extracellular peptidase activity on 0.1% gelatin zymograms of 3 QPX isolates (NY1, NY2, NY3) cultivated in minimal essential medium supplemented with gelatin hydrolysate, peptone, and yeastolate (each at 1 mg ml^{-1} final concentration). (A) Fresh extracellular product (ECP) without inhibitors; (B) ECP frozen for 7 d at −80°C without inhibitors; and (C) with 10 mM serine peptidase inhibitor PMSF

Differential gene expression of QPX peptidases

The expression of 1 subtilisin-like peptidase (S8-4) was differentially expressed among the 3 isolates (Fig. 4A), with significantly higher expression in the Peconic Bay isolate (NY3) as compared to the 2 Raritan Bay isolates (NY1 and NY2; Fig. 4A). In addition, the expression of 4 subtilisin-like peptidases varied among QPX cultures derived from different media, with generally a few-fold higher expression in QPX cells grown in MEM FBS. The higher expression of subtilisins in MEM FBS cultures corresponded well with the zymography results which revealed 1 subtilisin band only for that medium (compare Figs. 2A & 4B). However, gene regulation was statistically significant for only 1 subtilisin (S8-1, Fig. 4B), likely because of the high variability between the replicates.

Purification and LC/MS identification of a QPX peptidase

The peptidase activity band (35 kDa MW) that was inhibited by PMSF and suspected to be composed of subtilisins was chosen to be purified using ion exchange chromatography via FPLC. Proteins in FPLC fractions (12 to 19) were analyzed by SDS-PAGE (Fig. 5A), and peptidase activities in these fractions were tested on gelatin- and clam plasma-incorporated polyacrylamide gels (Fig. 5B,C). The thickest band (35 kDa) in fraction 19 was submitted for LC/MS analysis (Stony Brook Proteomics Center), which showed that the band contained a subtilisin type peptidase (S8-3; Fig. 6)

DISCUSSION

In silico characterization of QPX peptidases

We used bioinformatics tools to identify and describe peptidases possibly secreted by QPX, the thraustochytrid parasite infecting the hard clam *Mercenaria mercenaria*. Our investigation shows that 74 peptidases possess an N-terminal signal peptide sequence for the classical eukaryotic secretory pathway (Petersen et al. 2011). This number of putative QPX extracellular peptidases is relatively high but remains within the range described for other pathogenic microorganisms. For example, oomycete pathogens have been predicted to secrete between 18, for *Blastocystis* sp. (Denoeud et al. 2011), to over 90 peptidases for *Saprolegnia parasitica* (Jiang et al. 2013). Some of that variation is related to the type of bioinformatics tools used to predict extracellular proteins. For example, even though the SignalP v.4.1 tool shows that 74 QPX peptidases have the signal peptide in the beginning of the ORF, a different protein localization tool (Wolf PSORT) predicts only 62 QPX peptidases to be extracellular based on the same database of QPX ORFs (Rubin et al. 2015). Also, it is very likely that a different set of peptidases is secreted at any particular time depending on their need and function based on the substrate

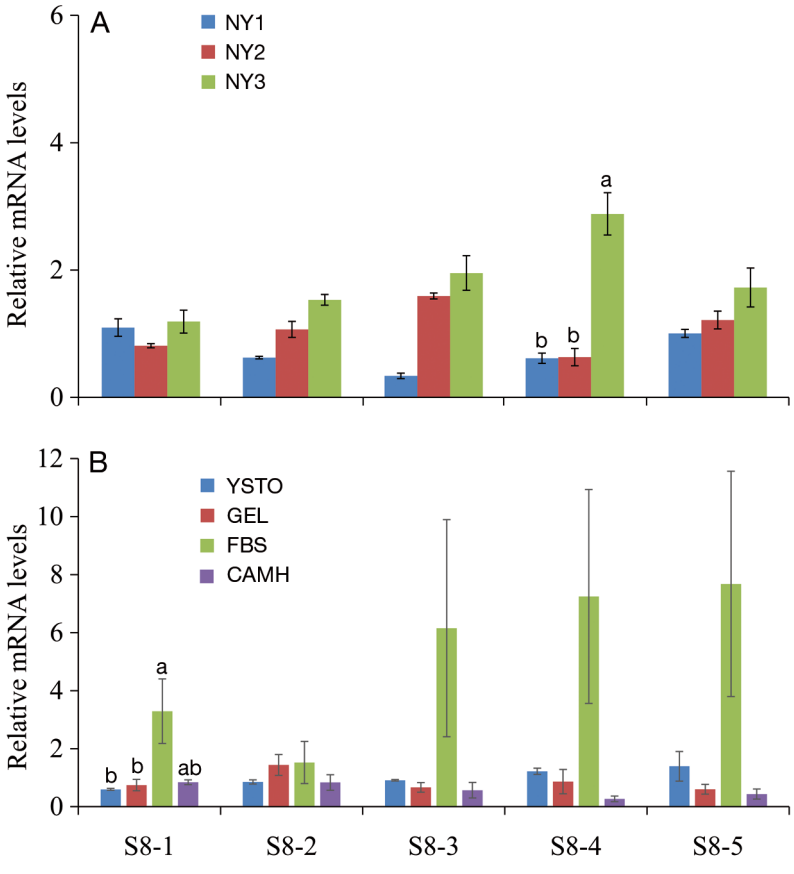

Fig. 4. Differential transcript levels of subtilisin-like (S8-1 to S8-5) peptidases in (A) QPX cells from 3 different isolates (NY1, NY2, and NY3) cultivated in minimal essential medium (MEM) supplemented with gelatin hydrolysate (GEL), yeastolate (YSTO), and peptone (1 mg ml^{-1} each, resulting in 3 mg ml^{-1} combined protein concentration), and in (B) QPX cells (isolate NY1) grown in MEM with an addition of 1 of 4 different protein supplements (all adjusted to final protein concentration of 1 mg ml^{-1}). Different letters (a,b) designate statistically significant differential gene expression (2-fold change; ANOVA, $p < 0.05$, Tukey post hoc analysis). FBS: fetal bovine serum; CAMH: clam adductor muscle homogenate

available for growth. In fact, only 8 peptidases were detected in QPX secretome by LC/MS (Rubin et al. 2015), but that study was conducted in QPX cells cultivated in 1 type of medium. Additional proteomics-based studies would be beneficial to test the number and identity of peptidases released by QPX cells under different culture conditions.

Our study also shows that the 74 QPX putative extracellular peptidases belong to 17 different families by the classification of the MEROPS database (Table 2). Members of at least 6 peptidase families including A1, S1, S8, S10, C1, and M35 are known virulence factors of pathogenic organisms (McKerrow et al. 1993, 2006, Klemba & Goldberg 2002, Monod et al. 2002). Since nothing is known about the virulence factors of labyrinthulomycetes, information on virulence peptidases from multiple eukaryotic and prokaryotic microorganisms has been used in the comparison below. In QPX cells, 3 peptidase families are represented by the highest number of transcripts: C1, the papain-like peptidase family; S8, the subtilisin-like peptidase family; and S10, also known as the serine carboxypeptidase family. Members of these 3 families are involved in pathogenicity or virulence in other microbial pathogens. For instance, the cysteine

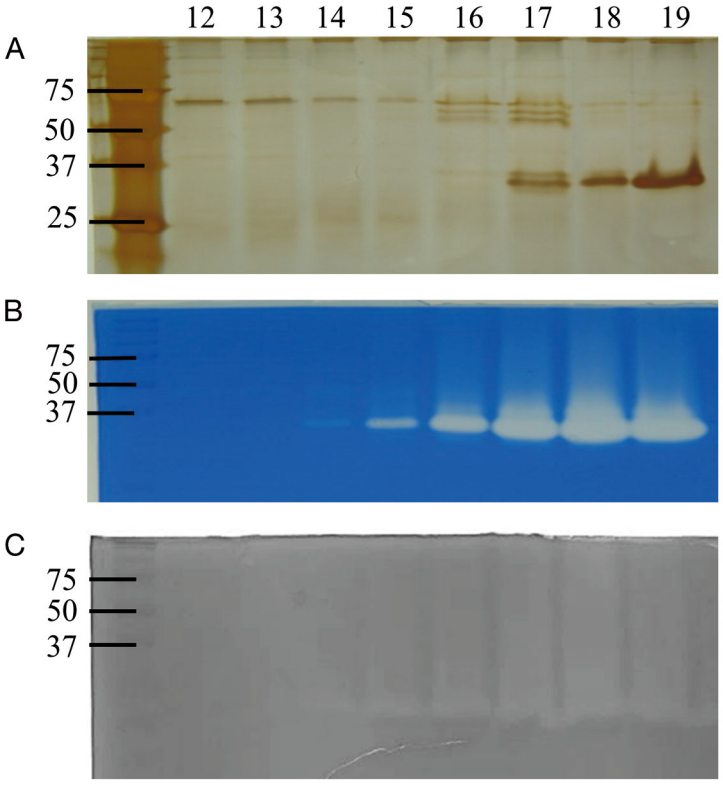

Fig. 5. (A) Fractions (12–19) of QPX extracellular product purified by ion exchange chromatography with protein content revealed on SDS-PAGE gel with silver nitrate stain, and (B,C) peptidase activity revealed on gelatin- and clam plasma- (pool from 3 clams) incorporated gels

QPX peptidase S8-3, length=400 aa
9 peptides, 14 spectra, 34.3% coverage

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  1 MVKLSLLLGG VTLFSKGANG MVGDETFEAH RRALSNSVVP DSYIVNAMVA
 51 DTAACDTLRN NIRQIQSQQN GLRAADTTLK SMRIKNNCFV RFKGPSASLP
101 TILSMEGVTD ISPEKFVYAA QGPESWGLDR IDQQDLPLNN LPFNSTHTGK
151 GVTVYILDTG VRGTHQEFAS RVQTGTSFVA GEGADFDGNG HGTHCAGIAT
201 GTTYGAAKEA TVIGVKVLSS SGSGTLSGVI EGIEWATNHA KSVGNMGVIS
251 MSLGGGRDSS TNAAVEAAAE EGMLVVVAAG NDNRNACFSS PASAGGKVVT
301 VGSTTISDER SWFSNYGTCV DIFAPGSDII SSWKDSDTST NTISGTSMAT
351 PFVAGVAATL LEKHNFDADA ARAELLTITA SSKLSDVGTG SPNKLLQTSR
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Fig. 6. Number of peptides identified by LC/MS for the QPX serine peptidase, subtilisin (S8-3)

peptidase family C1 (papain-like peptidases) is the most studied family in the context of virulence factors. Most known parasite-derived peptidase sequences belong to the C1 peptidase family (Atkinson et al. 2009). These include hemoglobinolytic falcipains from *Plasmodium* spp. and cruzain from *Trypanosoma cruzi* (Atkinson et al. 2009). Secreted C1 peptidases also determine pathogenesis of *Enteamoeba histolytica*, which uses these enzymes to digest the extracellular matrix of human tissue including collagen, elastin, fibrinogen, and laminin as well as immunoglobulin A (Que & Reed 2000). The S8 family, or subtilisins, are universal virulence factors of many pathogenic bacteria (Brown et al. 2000, Kennan et al. 2010, Bonifait et al. 2011), protozoa (Miller et al. 2001, Jean et al. 2003, Wanyiri et al. 2009, Swenerton et al. 2010, Hruzik et al. 2011), and fungi (Moser et al. 1994, Huang et al. 2004, Withers-Martinez et al. 2004). Subtilisins are also secreted by *Perkinsus marinus*, a protozoan pathogen of oysters, and are considered as important components of its virulence capabilities (Earnhart et al. 2004). Lastly, the S10 carboxypeptidases are secreted serine peptidases of some pathogenic fungi, including *Aspergillus niger* and *A. oryzae* (Monod et al. 2002) but are only lysosomal in pathogenic kinetoplastids such as *T. cruzi* (Parussini 2003, Alvarez et al. 2012).

Zymographic characterization of QPX peptidases

QPX was shown to secrete multiple extracellular peptidases, which were revealed by gelatin digestion incorporated in SDS polyacrylamide gels. A peptidase band of approximately 35 kDa MW was produced by all 3 QPX isolates and in all examined media, suggesting that it is composed of the primary peptidases used by QPX cells. Based on the effect of peptidase inhibitors, the band is composed mostly of subtilisin-type peptidases, as it was inhibited by PMSF and chymostatin, but not inhibited by other types of serine peptidase inhibitors. The inhibition assay was in agreement with the analysis by LC/MS of that band from NY3 isolate present in a purified FPLC fraction that showed the presence of 1 subtilisin. Three different subtilisins were also detected in the NY1 isolate in a previous mass spectrometry analysis of QPX secretion (Rubin et al. 2015).

Based on QPX transcriptome sequences, QPX is also predicted to secrete aspartate, cysteine, and metallo-peptidases, which were not revealed on the zymograms used in the study. Only 1 type of substrate was used, viz. gelatin, and the peptidase activity was developed in pH 8 buffer. Preliminary tests using different pH of the developing buffer showed no peptidase activity except for the slightly alkaline buffer. Gelatin is a commonly used substrate to detect multiple peptidases because it is composed of partially digested collagen and contains small peptides with different amino acid sequences (Lantz & Ciborowski 1994). However, additional peptidases might be revealed on gels containing other protein substrates such as casein, fibrinogen, and elastin (Bertolini & Rohovec 1992, La Peyre et al. 1995). It is also possible that the other peptidases are secreted in low quantities, not detectable by this technique. Previous LC/MS analysis of QPX secretion revealed an additional serine carboxypeptidase (S10), 2 papain-like peptidases (C1), and 2 metallopeptidases (M12B, M35) secreted by QPX cells cultivated in MEM supplemented only with yeastolate (Rubin et al. 2015).

The present study also shows that QPX cells produce different types and amounts of peptidases in response to different protein content in media. In general, QPX cells cultivated in MEM supplemented with a mix of gelatin hydrolysate, yeastolate, and peptone had overall higher extracellular peptidase production than QPX cells cultivated in MEM supplemented with FBS or CAMH. In addition, QPX cells cultivated in MEM supplemented with 3 or 4 mg ml^{-1} of FBS showed higher production of extracellular peptidases than cultures with 1 mg ml^{-1} of FBS, for which only 1 peptidase band was detected. In general, these results suggest that QPX is a well-adapted saprophytic organism secreting peptidases to use protein-rich organic matter of animal origin (collagen, peptone, bovine serum). It is thought that QPX can survive in the environment outside its host because it has been detected in some environmental samples including seawater, marine sediment, algae, and organic matter

scraped from shell surfaces of marine invertebrates (Gast et al. 2008). No studies have been conducted to investigate for how long QPX can survive without a sufficient protein source. Based on the data of peptidase activity from this study, it can be speculated that QPX cells can thrive on decomposing animal matter possibly including dead clam tissues.

Possible role of QPX subtilisins

Subtilisins are secreted when QPX is cultivated in clam adductor muscle homogenate (Fig. 2B), which indicates the ability of the parasite to digest major proteins present in muscular tissue (e.g. myosin). QPX also secretes subtilisins when cultivated in media containing collagen (gelatin; Fig. 3), suggesting that QPX cells can grow on connective tissues, which is in agreement with the microscopic localization of the parasite in infected clams (Dahl & Allam 2007). This is also in agreement with previous studies showing that extracellular subtilisins of other pathogenic organisms are able to digest gelatin (Bonifait et al. 2011) or have strong activity toward the extracellular matrix of animal connective tissue (Hong et al. 2000). The FPLC-purified fraction containing 1 QPX subtilisin peptidase was shown to digest some unidentified proteins in freshly collected clam plasma, suggesting that it might play an important role in QPX infection *in vivo*. It should be noted that the expression of subtilisin-type peptidases is most likely regulated at the transcriptional level, as differential expression was seen in QPX grown in various culture media even though large variability between replicates reduced statistical significance (only S8-1 regulation was statistically significant; Fig. 4B). These results highlight the need for additional investigations of QPX subtilisins as possible QPX virulence factors. Finally, the transcript levels of 1 subtilisin-like peptidase (S8-4; Fig. 4A) was differentially expressed among 3 different QPX isolates. The difference in the expression of that peptidase among different QPX isolates was also evident from the zymograms. The gene coding for the subtilisin-like peptidase S8-4, and specifically the 5′ untranslated region and its transcription factor binding site, can be investigated for the presence of nucleotide polymorphisms and its application for intraspecific studies of QPX.

CONCLUSIONS

In summary, this study demonstrates that QPX cells secrete many different peptidases. Based on transcriptomic information, the parasite is capable of producing over 70 extracellular peptidases. Half of these peptidases belong to 3 families: papain-like, subtilisin-like, and serine carboxy-peptidases, which are all known to contribute to the virulence of other pathogenic microorganisms. Several QPX peptidase activity bands can be revealed on gelatin incorporated SDS-PAGE. QPX produces different peptidases in response to varying protein composition and concentration in the growth media. Subtilisin-like peptidases are the main enzymes used by QPX cells as they were secreted in all studied media. Based on preliminary results, QPX subtilisins are very good candidates for further examination of molecular factors mediating QPX virulence.

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