JOURNAL OF PROTEOMICS 73 (2009) 209–217

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Proteomic clues to the identification of QX disease-resistance biomarkers in selectively bred Sydney rock oysters, *Saccostrea glomerata*

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ARTICLE INFO

Article history:
Received 2 September 2008
Accepted 23 June 2009

Keywords:
QX disease
Marteilia sydneyi
Proteomics
Saccostrea glomerata
Sydney rock oysters
Superoxide dismutase
Two dimensional electrophoresis

ABSTRACT

The Sydney rock oyster, *Saccostrea glomerata*, is susceptible to infection by the protozoan parasite, *Marteilia sydneyi*, the causative agent of QX disease. *M. sydneyi* infection peaks during summer when QX disease can cause up to 95% mortality. The current study takes a proteomic approach using 2-dimensional electrophoresis and mass spectrometry to identify markers of QX disease resistance among Sydney rock oysters. Proteome maps were developed for QX disease-resistant and -susceptible oysters. Six proteins in those maps were clearly associated with resistance and so were characterized by mass spectrometry. Two of the proteins (p9 and p11) were homologous to superoxide dismutase-like molecules from the Pacific oyster, *Crassostrea gigas*, and the Eastern oyster, *Crassostrea virginica*. The remaining *S. glomerata* proteins had no obvious similarities to known molecules in sequence databases. p9 and p11 are currently being investigated as potential markers for the selective breeding of QX disease-resistant oysters.

1. Introduction

QX disease is caused by the protozoan parasite, *Marteilia sydneyi*. The parasite causes epizootics among Sydney rock oysters (*Saccostrea glomerata*) that occur during summer and can result in up to 95% mortality. The disease is more common in the northern, warmer estuaries on Australian's eastern seaboard, in the states of New South Wales (NSW) and Queensland. QX was responsible for a decline of the oyster industry in southern Queensland and in the Tweed, Richmond and Clarence Rivers of northern NSW during the 1970's [1]. The disease was later identified around Sydney, in the Georges River in 1994 and in the Hawkesbury River in 2004. These most recent outbreaks have had devastating effects on rock oyster production, which declined by 80% between 1994 and 2000 in Georges River, and was largely abandoned on many areas of Hawkesbury River within 18 months of first outbreak in 2004 [2].

M. sydneyi was initially characterized by Wolf in 1972 [3], and Perkins and Wolf in 1976 [4]. Oysters that are heavily infected with *M. sydneyi* are usually in poor condition with their gonads completely resorbed [2]. Infection results in death from starvation less than 60 days after the initial infection. The parasite invades digestive gland epithelial cells, leading to complete disorganization of the infected gut tissues [5–7]. *M sydneyi* sporonts pass into the environment via the alimentary

Abbreviations: 2DE, two dimensional electrophoresis; DPI, Department of Primary Industries; NSW, New South Wales; QXR5, fifth generation oysters bred for QX disease resistance; QXR6, sixth generation oysters bred for QX disease resistance; SOD, superoxide dismutase; WB, fifth generation multidisease-resistant oysters from Woolooware Bay.

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doi:10.1016/j.jprot.2009.06.012

canal before the death of the oyster [8]. A variety of developmental stages of the parasite in *S. glomerata* have been characterized by Kleeman et al. [9,10], and a putative intermediate host for the parasite has recently been identified (R. Adlard, Queensland Museum, personal communication).

In the 1990's, the New South Wales Department of Primary Industries (NSW DPI) began a rock oyster breeding program for fast growth and resistance to Winter Mortality Syndrome. Winter mortality is caused by the protozoa parasite, *Bonamia roughleyi*, which can kill up to 80% of oysters [11]. In 1994, an outbreak of QX disease in the Georges River, Sydney, caused 85% mortality in 2 out of the 3 breeding lines established by NSW DPI. As a result, the program was altered in 1997 to include breeding for QX disease resistance. After 2 generations, the mortality rate from QX disease was reduced to 62% in the best QX resistance (QXR) breeding lines. It was further decreased to 50% in fourth generation (QXR_4) and to 30% in fifth generation resistant (QXR_5) oysters [11–13; and unpublished data].

Our laboratory has been investigating the role of the defensive enzyme, phenoloxidase, as a potential marker of QX disease resistance. In 2003, we showed that *M. sydneyi* infects oysters when their phenoloxidase system is suppressed [14]. Phenoloxidase is not affected in rivers that do not suffer QX disease outbreaks. However, in oysters transferred from non-infested to QX-infested rivers, phenoloxidase activity decreases rapidly immediately before oyster are infected by *M. sydneyi* [14]. We concluded that the suppression of phenoloxidase-mediated defense decreases the ability of oysters to control *M. sydneyi*, leading to the development of QX disease. The implication of phenoloxidase in QX disease has been further supported by studies of the QX-resistant oysters being bred by NSW DPI. The breeding program has selected oysters with enhanced phenoloxidase activities and novel forms of the enzyme [15,16].

Although QX resistance breeding has reduced mortality, one remaining limitation in the breeding program is that genetic factors, in addition to phenoloxidase, that might enhance disease resistance during selection have not been identified. This continues to limit the scope for marker-assisted selection. Despite the potential of phenoloxidase as a marker of QX disease resistance, NSW DPI and the Sydney rock oyster industry are eager to identify additional genetic factors that contribute to disease resistance. Here, we use proteomics to identify new markers of QX disease resistance. This is the first time that proteomics has been employed to study the expression of proteins in *S. glomerata*. Differences in the proteomes of wild-type, QXR and *M.* sydneyi-infected oysters are examined using 2-dimensional electrophoresis (2DE) to identify proteins that differ in expression between oyster populations. These proteins are then characterized by mass spectrometry.

2. Experimental procedures

2.1. Oysters

Four types of *S. glomerata* were used in this study; uninfected wild-type oysters, *M. sydneyi*-infected wild-type oysters and two strains of selectively bred oysters. Wild-type *S. glomerata* are commercially farmed oysters that have not been selected for disease resistance. They were purchased from the Sydney Fish Markets. Disease-resistant oyster lines were derived from successive generations of selective breeding in which parental broodstock had survived disease outbreaks [12,13] The original broodstock for the first generation of selective breeding came from the wild-type population. Their descendents constitute distinct breeding lines, two of which were used in this study. These were the QXR line from Lime Kiln Bar on Georges River, which was bred specifically for QX disease resistance [13 and unpublished data], and the Woolooware Bay (WB) line, which has been selected for resistance to both QX disease and Winter Mortality Syndrome [13 and unpublished data]. Within the QXR line, QXR_5 oysters were from the fifth generation of selection and QXR_6 were from the sixth generation. QXR, wild-type and WB oysters were maintained for at least 20 days at Macquarie University in 30 L aquaria filled with UV-sterilized re-circulating oceanic seawater. The oysters were fed periodically with Aquasonic invertebrate food supplement (5 mL/200 L water) and exposed to identical growing conditions. None of these oysters had been exposed to active QX disease infections. An additional group of wild-type oysters showing symptoms of QX disease were obtained from the Macleay River (MR) NSW. These wild-type oysters had active QX disease infections. Prior to proteomic analyses, all oysters were screened for the presence of *M. sydneyi* spores in their digestive glands by the method of Kleeman and Adlard [10]. With the exception of the wild-type oysters from the MR, none of the other oysters showed the presence of parasite spores (i.e., they were free of QX disease).

2.2. Collection of hemolymph

Oysters were removed from aquaria 30 min prior to hemolymph extraction so that excess seawater could drain from their mantle cavities. Oysters were then shucked and the exuding hemolymph was removed from the mantle cavity. Approximately 3 mL of hemolymph was collected from each oyster. Hemolymph was immediately transferred to 10 mL polypropylene tubes containing an equal volume of marine anticoagulant (MAC, 0.1 M glucose, 15 mM trisodium citrate, 13 mM citric acid, 10 mM EDTA, 0.45 M NaCl, pH7.0). Protease inhibitor 10 μL (Mini Cocktail Tablets EDTA-free, Boehringer Mannheim, Germany) was added before the hemolymph was stored at –80 °C.

The total hemolymph protein concentrations of samples were determined using the Bradford assay with a Bio-Rad Protein Assay Kit (Bio-Rad, Castle Hill, NSW). Bovine serum albumin was used to generate standard curves. The density of the hemocytes in hemolymph and the frequency of *M. sydneyi* in infected oysters were determined using an improved Neubauer hemocytometer.

2.3. Two dimensional electrophoresis (2DE)

Protein precipitation and quantification were performed on whole hemolymph using 2-D Clean-Up kits and 2-D Quant kits according to manufacturer's instructions (Amersham Biosciences, NJ, USA). Two hundred micrograms of precipitated proteins was re-suspended in 125 μg of rehydration buffer containing 8 M urea, 2% 3-cholamydopropyl dimethyl-

ammonio, 1-propane sulphate (CHAPS), 2.8 mg dithiothreitol (DTT), 0.002% w/v bromophenol blue and 0.5% v/v 3–10 pharmalyte or IPG buffer (Bio-Rad, Castle Hill, NSW).

Samples were subject to 2DE as described by O'Farrell et al. [17,18]. Proteins were separated by isoelectric focusing (IEF) using 7 cm immobilized pH gradient strips (IPG strips; pH 3–10; Bio-Rad). The IPG strips were rehydrated overnight with the protein samples that had been prepared in rehydration buffer. Isoelectric focusing was performed using an IPGPhor Isoelectric Focusing Cell (Amersham Biosciences). After isoelectric focusing, the IPG strips were equilibrated for 15 min in 10 mL reducing buffer (6 M urea, 0.375 M Tris–HCl pH 8.8, 2% w/v SDS, 20% v/v glycerol, 2% w/v DTT, 1% bromophenol blue) and for a further 15 min in alkylation buffer (6 M urea, 0.375 M Tris–HCl pH 8.8, 2% w/v SDS, 20% v/v glycerol, 2.5% w/v idodoacetamide, 1% w/v bromophenol blue). Second dimension SDS-PAGE was performed on 15% polyacrylamide gels for approximately 2 h using Mini Protean II electrophoresis cells. All gels were loaded with 2D SDS-PAGE standards or broad range SDS-PAGE molecular weight markers (Bio-Rad) as required.

After 2DE, protein spots were visualized by silver staining according to the method of Blum et al. [19]. Gel images were captured in tagged image file format (TIFF) with a UMAX PowerLook III flatbed scanner (UMAX Technologies, Dallas, TX, USA) at 600 dpi. The images were analysed using Progenesis image analysis software package (Nonlinear Dynamics, PG 240, version 2006, UK) [20]. After automatic spot detection, spot boundaries were manually edited and artefacts on the images were removed. All spot volumes were normalized using built-in algorithms. Normalized spot intensities, isoelectric points (pIs) and MW data were obtained for each protein spot on the images. Each sample was analysed in triplicate and a composite image of the three gels was generated. Gel images from the each oyster line were grouped as single experiments. Comparisons of spots on different gels were then performed as indicated below.

2.4. *Mass spectrometry*

Proteins of interest were excised from gels using sterile scalpel blades. Excised proteins were de-stained by soaking in 100 mM sodium thiosulfate and 30 mM potassium ferricyanide for 20 min before being washed with distilled, deionised water (4×250 μL). After de-staining, the proteins were digested with 20 μL of trypsin (20 μg/mL in 100 mM ammonium bicarbonate, Promega, WI.) at 37 °C overnight. The resulting tryptic peptides were extracted from the gel slices by washing with distilled, deionised water (50 μL), followed by 2 washes with 75 μL 5% formic acid in 50% acetonitrile and one wash in 100% acetonitrile with the aid of sonication. The extracts were combined and concentrated to 10 μL by vacuum centrifugation.

Electrospray ionization tandem mass spectrometry (ESI MS/MS) was performed at the Bio Molecular Sequencing Facility (BMSF; University of NSW) using LTQ FT Ultra Hybrid Mass Spectrometer (Thermo Finnigan). Peak lists were generated using Mascot Daemon/extract_msn (Matrix Science, London, England, Thermo) using default parameters and submitted to the database search program Mascot (version 2.1, Matrix Science). Search parameters were: precursor tolerance ±1.4 Da and product ion tolerances ±0.6 Da; Met(O) and Cys-carboxyamidomethylation specified as variable modification, enzyme specificity was trypsin, 1 missed cleavage was possible and the NCBInr database (15_7_07; 5303346 sequences; 1837221997 residues; September 2007) was searched. Individual ions scores >56 indicated identity or extensive homology ($p<0.05$). No species restrictions were applied for homology searches.

2.5. *Statistical analysis of protein expression data: Non-metric multidimensional scaling*

Non-metric multidimensional scaling (MDS) was employed to construct a numerical "map" of combined expression data for individual oysters using PRIMER v5 analysis software (PRIMER-E Ltd, Plymouth, 2001) [21]. Differences in proteomic profiles of QXR, wild-type and WB oysters were used to generate Bray–Curtis dissimilarity matrices according to the following formula:

$$S_{jk} = 100\left\{1 - \frac{\sum_{i=1}^{p} |y_{ij} - y_{ik}|}{\sum_{i=1}^{p} |y_{ij} + y_{ik}|}\right\}$$

where j,k represent protein intensities in the oyster samples under comparison and y represents the normalized, double root transformed protein abundance values. As some of the proteins expressed in the hemocytes' proteomes were highly abundant, double root transformation of the raw data was employed to down-weight the relative contribution of these proteins to the other, less abundant proteins for the Bray–Curtis similarity calculations [21].

Non-metric MDS provides a visual representation of the protein expression profiles. The Bray–Curtis dissimilarity coefficients obtained for the protein expression data (see above) were used for this analysis. The MDS algorithm computes a relationship between the expression values in multidimensional space and their Bray–Curtis dissimilarity coefficients. The scatter of these values from the computed regression provides a goodness-of-fit measure (stress) for each data point and is calculated according to the following formula:

$$Stress = \sqrt{\frac{\sum_j \sum_k (djk - \hat{d}jk)^2}{\sum_j \sum_k \hat{d}_{jk}^2}}$$

where $\hat{d}_{jk}$ represents the distance between the points j and k as measured by the regression line corresponding to the Bray–Curtis dissimilarity δ_{jk}. Stress values of <0.1 indicate good ordination in expression space, while values of <0.05 are highly significant and represent true representation of dissimilarities in expression space [21].

In these plots, larger bubble sizes indicate greater expression levels of the superimposed variables. The distance between bubbles indicates how closely individual oysters were related to each other in multidimensional plains, where each of the dimensions represents the expression of one of the six resistance-associated proteins, or phenoloxidase activity [21].

Cluster analyses were also performed on the same data set. Group average linkage in a hierarchical, agglomerative

clustering algorithm was performed by PRIMER to plot dendrograms. The dendrograms indicated the distance between clustered groups and sub-groups in the different populations (uninfected wild-type, infected wild-type, QXR_5, QXR_6 and WB lines) [21].

2.6. *Other statistical analysis*

Differences in mean values for individual factors were tested for statistical significance either by using the ANOVA method (Microsoft Excel with Pop Tools addin) or by the Mann–Whitney test, accessed via Vassarstats (http://faculty.vassar.edu/lowry/VassarStats.html).

3. Results

3.1. *Hemolymph protein concentrations*

The hemolymph protein concentrations of wild-type and QXR_5 oysters were compared using a one-way analysis of variance (ANOVA). Total protein concentrations in the wild-type population were found to be significantly higher than those of the QXR_5 population ($df=1$, $n=80$, $p<0.05$). Wild-type oysters had approximately 25% more protein in their hemolymph than QXR_5 oysters (Fig. 1).

3.2. *Proteome maps of wild-type, QXR and WB oysters*

Fig. 2 shows a representative proteome map of individuals from the QXR_5 population, as well as uninfected wild-type and *M. sydneyi*-infected wild-type oysters. The initial experiments focused on the wild-type (infected and uninfected) and QXR_5 oysters to identify proteins of interest. These proteins were then analysed in other oyster lines (QXR_6 and WB, see below). In total, five proteome maps of total hemolymph proteins from different QXR_5 individuals were compared with five maps from uninfected wild-type and five maps from infected wild-type oysters. Image analysis (Progenesis) detected 60 protein spots in proteome maps of QXR_5 oyster hemolymph, 80 spots in uninfected wild-type oysters, and 140 spots in QX-

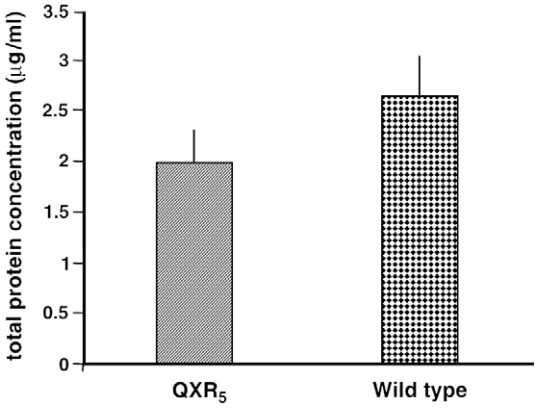

Fig. 1 – Total protein concentration of hemolymph (µg/mL) from uninfected wild-type and QXR_5 oysters. $n=40$ from each population, bars = SEM.

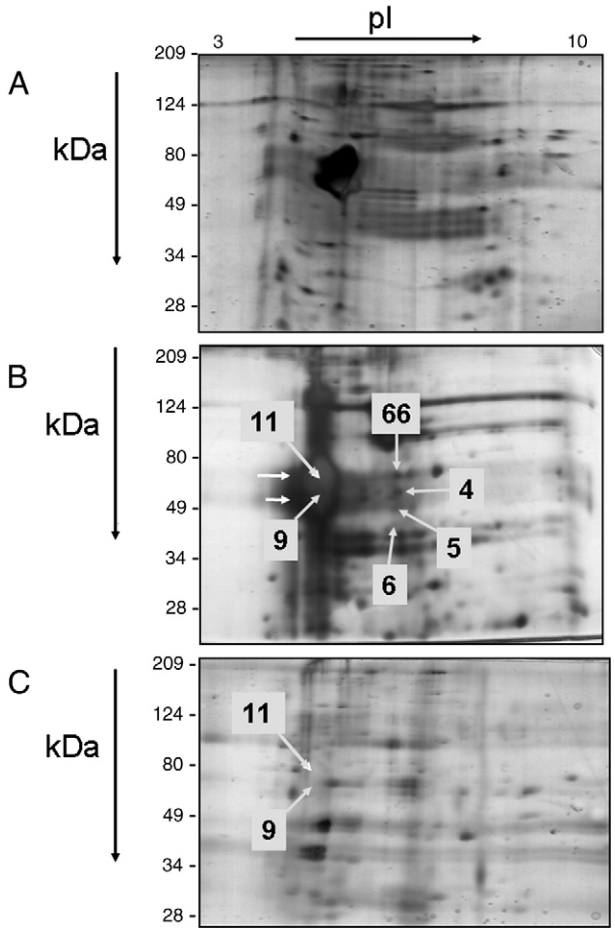

Fig. 2 – Proteome maps of (A) uninfected wild-type, (B) QXR_5 and (C) *M. sydneyi*-infected wild-type oyster hemolymph proteins. Arrows in (B) indicate proteins present primarily in QXR_5 oysters. Arrows in (C) show the expected position of proteins p9 and p11.

infected wild-type oysters (Supplementary Table 1). Spots that differed in frequency and intensity between populations and within the populations were further evaluated. An additional nine proteome maps of hemolymph proteins from QXR_6 oysters and five from WB individuals were also studied (Supplementary Table 1).

Six protein spots with molecular weights ranging from 48 to 68 kDa and pIs from 5.6 to 6.7 were expressed more frequently and at higher concentrations in QXR_6 oysters compared to uninfected, wild-type oysters (Fig. 2B). For each protein, its mean expression intensities differed significantly between the QXR_6 and wild-type population ($p<0.05$). Two of these spots, p9 (49 kDa, pI 5.6) and p11 (60 kDa, pI 5.7), were expressed in all oyster strains, but had higher expression levels in both QXR lines and WB oysters than in uninfected, wild-types (Fig. 3A and B). Fig. 3A shows that four QXR_5 oysters expressed p11 at higher intensities than the five wild-type individuals. The maximum expression intensity of p11 among these four QXR_5 oysters was 15.7 and the minimum was 10.9, while the maximum intensity of p11 expression in wild-types was 8.7. One of the QXR_5 oysters analysed had a level of p11 expression comparable to that of wild-type oysters (relative

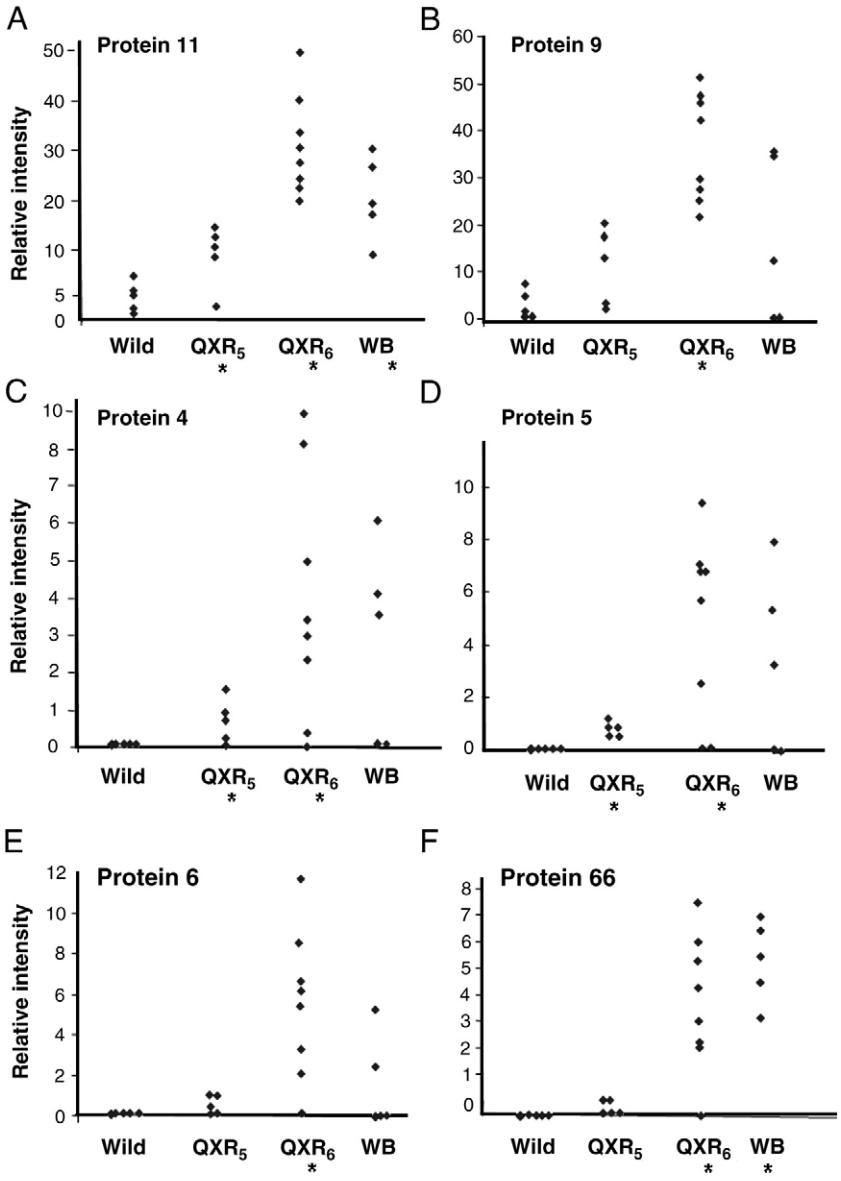

Fig. 3 – Relative expression levels for the six proteins (p4, p5, p6, p9, p11 and p66) that were associated with QX disease resistance in the uninfected wild-type, QXR_5, QXR6, and WB populations. Each (♦) represents expression data for a single oyster. * indicates oyster lines whose protein expression levels are significantly different to WT (wild-type) oysters ($p<0.05$).

intensity 3.1). The expression of p11 in four out of five WB oysters was higher than in QXR_5 oysters, and QXR_6 had higher levels of p11 than all other oyster strains ($p<0.05$).

The expression of p9 in three out of five QXR_5 oysters was greater than that of wild-type oysters. These three QXR_5 individuals expressed p9 with an average intensity 16.8±2.7 compared to 2.4±1.5 in the five wild-type oysters ($p<0.05$). p9 was entirely absent in two wild-type oysters. The remaining two QXR_5 oysters had expression levels for p9 (relative intensities 2.3 and 3.4) that fell within the range of the wild-type oysters (0–4.2). QXR_6 oysters expressed p9 at much higher intensities compared to the other oyster strains ($p<0.05$) with a maximum intensity in QXR_6 oysters of 51.2 and a minimum of 24.3. Only three out of five WB oysters expressed detectable levels of p9 (34.4 and 35.46) (Fig. 3B).

Both p11 and p9 were entirely absent in all five of the infected wild-type oysters tested (Fig. 2C).

The other four protein spots that differed in expression between QXR and wild-type oysters were p4 (65 kDa, pI 6.68), p5 (58 kDa, pI 6.69), p6 (48 kDa, pI 6.68) and p66 (68 kDa, pI 6.69). These protein spots were only present in QXR oysters and were not expressed by any of the uninfected or infected wild-type oysters tested. The frequency with which they appeared and their intensity varied substantially within the QXR_5 population (Fig. 3). Fig. 3 shows that p4 was expressed by 4 out of 5 QXR_5 oysters, while p5 was present in all five QXR_5 individuals. p6 was expressed by three out of five QXR_5 individuals and p66 was present in only two of the QXR_5 individuals.

The QXR_6 population expressed p4, p5, p6 and p66 at much higher intensities than QXR_5 and WB oysters. Six oysters from

QXR_6 expressed p4 and p5 at higher intensities compared to all five QXR_5 oysters, with relative expression ranging from 2.3 to 10.1 for p4 and 2.5 to 9.6 for p5. Only three out of five WB oysters had expression levels of p4 and p5 that fell within the range of the QXR_6 individuals. The remaining two WB oysters did not express p4 and p5 (Fig. 3C and D and Supplementary Table 1). Seven QXR_6 oysters expressed p6 and p66 at higher intensities compared to all five QXR_5 oysters, with relative expression ranging from 2.1 to 12.0 for p6 and 2.1 to 7.4 for p66. Only two of WB oysters expressed detectable quantities of p6 (Fig. 3E and F and Supplementary Table 1). Although the differences described here are statistically significant, the small sample sizes used in this study necessitate the extension of this characterization in more individuals.

3.3. *Mass spectrometry*

The protein spots p11, p9, p4, p5, p6 and p66, which were associated with QX disease resistance, were subjected to mass spectrometry. p11 was found to incorporate a tryptic peptide that was homologous to a protein from the Pacific oyster, *C. gigas*, designated cavortin (Table 1; Supplementary Table 2). Three ion fragments (p11.1, p11.2 and p11.3) matched a predicted tryptic peptide of 2492 Da from cavortin that was bounded by two R/S tryptic proteolysis sites. An additional ion fragment from p11 (p11.4) matched a slightly larger (2924 Da) predicted peptide from *C. gigas* cavortin that incorporated an additional three amino acids (HAH) on the carboxyl end of the 2492 Da peptide (Table 1; Supplementary Table 2).

Two tryptic fragments of p9 (p9.1 and p9.2) matched a predicted peptide from an uncharacterized EST from the Eastern oyster, *C. virginica*, designated, dominin (Genbank non-redundant database accession number BAF30874; Itoh et al., unpublished data). The matching predicted peptide from dominin had a molecular weight of 2492. It was identical in amino acid sequence to the cavortin peptide that matched p11, except for the presence of an isoleucine instead of a valine at reside 15. The remaining *S. glomerata* proteins (p66, p4, p5 and p6) had no obvious similarity to any known proteins in sequence databases.

3.4. *Non-metric multidimensional scaling (MDS)*

Fig. 4A shows a 2-dimensional MDS plot, in which pooled expression data for all six resistance-associated proteins (p11, p9, p66, p4, p5 and p6) and phenoloxidase activity, are shown for individual oysters (stress = 0.05). Distinct clusters representing the different populations are evident on the plot. These clusters agree with those generated by the matching cluster analysis (Fig. 4B). Wild-type oysters formed a relatively dispersed cluster to the right of the MDS plot. Two oysters ($QXR_5$1 and $QXR_5$5) fell within the region of the wild-type cluster. The other three QXR_5 oysters formed a discrete group between the wild-type and QXR_6 populations. Cluster analysis also grouped $QXR_5$1 and $QXR_5$5 with the wild-type oysters, whilst $QXR_5$2, $QXR_5$3 and $QXR_5$4 were grouped with the QXR_6 oysters. The two QX-resistant populations still formed discrete clusters, with QXR_5 clearly separated from the QXR_6 population. The larger "bubble" sizes of QXR_6 oysters relative to QXR_5 and wild-type oysters also reflects their increased expression of the resistance-associated proteins. Two of the oysters from the WB line (WB2 and WB3) clustered closely with the QXR_6 population, whilst the remaining two WB individuals were distinct from all of the other populations tested.

Fig. 4C shows the same type of MDS analysis for six randomly chosen proteins other than the resistance-associated proteins, p4, p5, p6, p9, p11 and p66,. The proteins selected for this "control" plot were not obviously associated with QX disease resistance. No clear clustering was evident in this plot. QXR_5 and wild-type oysters were equally dispersed (stress = 0.07) and cluster analysis failed to identify any discrete populations (data not shown).

Table 1 – Mascot search results for MS/MS analysis of *S. glomerata* proteins p9 and p11, showing the m/z (mass-to-charge) ratios and the observed (calculated) molecular weights (Mr observed) for four ion fragments from p11 and two fragments from p9.

Ion fragments from p11	m/z	Mr (observed)	Mr (expected)	Score	*E* value	Matching peptide sequences
						Best match: *Crassostrea gigas* cavortin (gi|30039400; Mass: 19276); Mascot Score: 111; Significant score cutoff: >56; Coverage: 16.7%
p11.1	832.0222	2493.044	2492.2627	37	3.5	R.SLAILQGDHTSHTAVIACCVIGR.S + Acrylamide(C);C
p11.2	1247.731	2493.447	2492.2627	57	0.036	R.SLAILQGDHTSHTAVIACCVIGR.S + Acrylamide(C);C
p11.3	832.2092	2493.605	2492.2627	28	23	R.SLAILQGDHTSHTAVIACCVIGR.S + Acrylamide(C);C
p11.4	976.2299	2925.667	2924.4497	54	0.053	R.SLAILQGDHTSHTAVIACCVIGRSHAH. + Acrylamide(C)
Ion fragments from p9	m/z	Mr (observed)	Mr (expected)	Score	*E* value	Matching peptide sequences
						Best match: *Crassostrea virginica* dominin (gi|113928362 Mass: 21042); Mascot Score: 94; Significant score cutoff: >56; Coverage: 13%
p9.1	1247.6580	2493.301	2492.2627	94	7.2e−6	R.SLAILQGDHTSHTAIIACCVIGR.S + 2(C)
p9.2	832.1196	2493.337	2492.2627	69	0.002	R.SLAILQGDHTSHTAIIACCVIGR.S + 2(C)

Also shown are the expected molecular weights (Mr expected) and amino acid sequences of the closest matching peptides to the *S. glomerata* fragments in the NCBInr database.

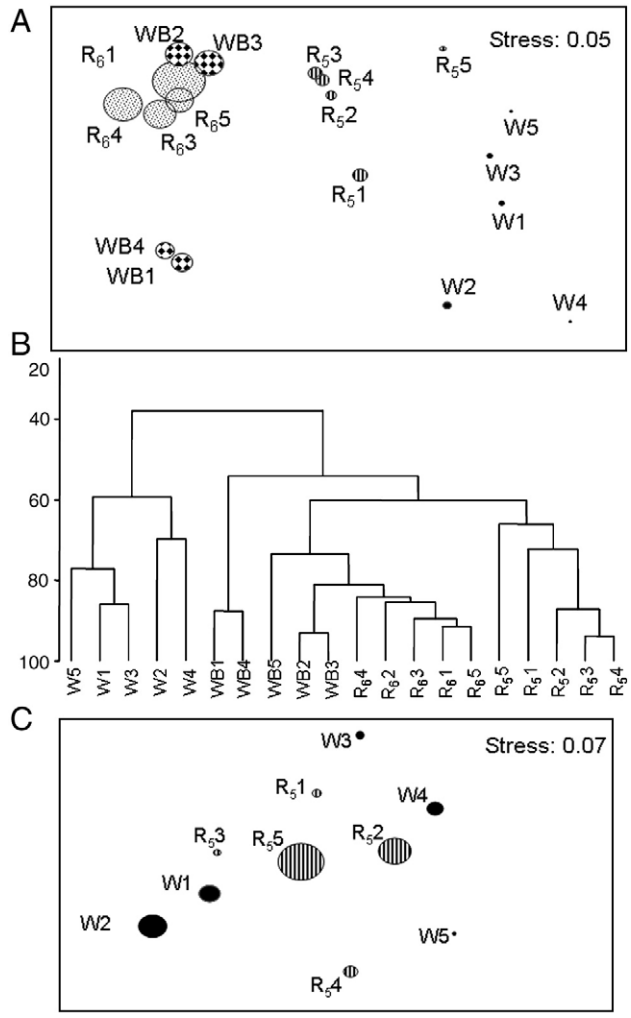

Fig. 4 – (A) Non-metric MDS bubble plot of combined expression data for six proteins associated with QX disease resistance in the uninfected wild-type, QXR_5, QXR_6, and WB populations (stress = 0.05). Each bubble represents data for a single oyster. (B) Dendrogram of combined expression data for six proteins associated with QX disease resistance in uninfected wild-type, QXR_5, QXR6, and WB populations. (C) Non-metric MDS bubble plot of combined expression data for six randomly chosen proteins (not obviously associated with resistance) in the QXR_5 and wild-type populations (stress = 0.07). W = uninfected wild-type, $R_5 = QXR_5$, $R_6 = QXR_6$, WB = Woolooware Bay line.

4. Discussion

The productivity of Sydney rock oyster farming in Australia has fallen by over 40% in the past 30 years [22]. This decline has resulted in part by two infectious diseases, Winter Mortality Syndrome and QX disease [23]. Selective breeding undertaken by NSW DPI has reduced the mortality rates of both diseases. However, the introduction of marker-assisted selection is still required to increase the efficiency of the breeding program and to maintain resistance in the absence of high level selection in the field.

This study employed proteomics to identify new markers of QX disease resistance. Our aim was to identify proteins that are differentially expressed between wild-type oysters and selective breeding lines. It was immediately clear that overall protein expression differed between the populations. Total hemolymph protein concentrations were found to be significantly higher in wild-type oysters when compared to the resistant strain. This agrees with our proteomic analysis of the two populations, which identified more proteins on proteome maps of wild-type oysters compared to QXR animals. However, it is unlikely that the observed differences in protein expression between the oyster lines were due to inbreeding during the cultivation of QXR oysters. Preliminary genetic diversity analyses of third generation mass selected oysters using allozyme markers [29] or microsatellite markers of the sixth generation of QXR oysters (as used in this study) found no evidence of significant inbreeding (Wayne O'Connor, unpublished data). Hence, the differences in protein expression may be attributed to the selective breeding for QXR disease resistance and not inbreeding.

To identify difference in the constitutive expression of proteins between wild-type oysters and the selective breeding lines, we tested oysters from each population that had been held under the same environmental conditions in the laboratory. Under these conditions, differences between the populations are most likely the result of genetic changes associated with selective breeding. Proteome maps based on 2DE data showed remarkable consistency between individuals of oyster lines used in this study (i.e. wild-type, QXR, infected). For example, the proteome maps generated from the blood cells from QXR oysters showed the normalized intensities of proteins spots were highly similar between individuals, with an average of 5% of protein spots showing intensities greater than one standard deviation of mean spot intensities (5 ± 3%, data not shown). Even though the samples sizes used in this study are relatively low ($n = 5–8$ oysters per population), this homogeneity within population meant that statistically significant difference could be identified in protein expression between populations. We analysed the proteome maps with a view to identifying protein spots that significantly differed in their expression frequency or intensity between the wild-type and QXR populations. 2DE showed that six proteins with molecular weights ranging from 49 to 65 kDa and pIs from 5.6 to 6.69 were specifically associated with resistance breeding. Two of those proteins, p11 (60 kDa, pI 5.7) and p9 (49 kDa, pI 5.6) occurred more frequently and at greater intensity in QXR oysters compared to wild-types. Interestingly, not all QXR_5 oysters expressed p9 and p11 at enhanced levels compared to wild-types. This suggests that there is still heterogeneity among the genes that encode these proteins in QXR_5 population, which fits with expectations for QX resistance-associated molecules. Current mortality data indicate that only 50%–60% of the QXR_5 populations have genetic resistance to the disease and so resistance-associated molecules should not be found in all QXR_5 individuals (J Nell unpublished data). Similar heterogeneity was not evident among the QXR_6 oysters tested in this study, all of which expressed p11 and p9 at high levels. This may be because QXR_6 oysters are the progeny of QXR_5 oysters that had survived QX disease outbreaks, and so would be expected to have higher frequencies of disease resistance genes.

The concentrations of p9 and p11 also varied between the two selective breeding regimes tested here. In QXR oysters, which were bred specifically for QX disease-resistance, these proteins were more frequent and present at higher titers than oysters from the WB line, bred for combined Winter Mortality and QX disease-resistance. This matches expectations based on mortality rates for the QXR_5 and WB populations. According to the latest NSW DPI data, QX disease associated mortality rate for QXR_5 in Georges River is lower than that of the WB line (J Nell unpublished data). Most significantly, p9 and p11 were entirely absent from infected wild-type oysters (Fig. 2C), which may indicate a direct association between these proteins and infection by *M. sydneyi*, the etiological agent of QX disease.

ESI MS/MS showed that p9 and p11 incorporate peptides that closely resemble regions of the proteins cavortin and dominin from the Pacific oyster, *C. gigas*, and the Eastern oyster, *C. virginica* (Table 1). Both cavortin and dominin are closely related to superoxide dismutases (SODs) [27], suggesting that p9 and p11 from *S. glomerata* incorporate SOD-like domains. The SOD-like domains of the *Crassostrea* proteins have a copper binding site with sequence similarities to SODs from other species that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. This is an important antioxidant defense mechanism in almost all cells, including those of mollusks [24]. SODs in most eukaryotic cells exist as dimeric copper or zinc containing proteins [25]. However the SOD-like domains of cavortin and dominin are highly modified with numerous insertions and deletions, which suggest that they may have modified enzymatic activities relative to true SODs [24].

Summer mortality has been reported in the Pacific oyster, *C. gigas*, for many years and is mainly associated with high temperature and the oyster reproductive period [26]. A previous study of *C. gigas* by Huvet et al. showed that cavortin is associated with resistance to summer mortality. The protein is more highly expressed in oysters that are resistant to summer mortality compared to susceptible animals [27].

One intriguing possibility from our results is that, in *S. glomerata*, SOD-like proteins have adopted phenoloxidase-like enzymatic activities or otherwise contribute to oxidative processes associated with parasite killing. Our previous work has found a strong association between QX disease resistance and the phenoloxidase system, and that phenoloxidase activities are higher in QXR oysters compared to the susceptible population [14,15,28]. The potential role of the proteins p9 and p11 in oxidative reactions fits well with this known resistance mechanism. We are currently investigating the potential p9 and p11 as genetic markers of QX disease-resistance, and further studying their relationship to the phenoloxidase activities that are known to be associated with disease resistance.

The remaining four QX resistance-associated proteins detected in *S. glomerata*, p4 (65 kDa, pI 6.687), p5 (58 kDa, pI 6.69), p6 (48 kDa, pI 6.68) and 66 (68 kDa, pI 6.69), were present only in QXR and WB oysters, with different concentrations within each population. Given that mortality data predict the appearance of QX disease susceptible oysters in both the QXR_5 and WB lines, their expression in all resistant oysters suggests that these proteins may not be reliable markers of disease resistance when compared to p11 and p9. Having said this, the relatively low sample sizes used in this study warrant more extensive analyses of these proteins before definitive conclusions can be made about their usefulness as genetic markers for selective breeding.

In this study, the most effective predictor of QX resistance came from combining expression data for all six resistance-associated proteins. MDS and associated cluster analysis revealed clear distinctions between different oyster populations. In MDS bubble plots, significant distances were evident between the wild-type oysters and all of the resistant lines. However, two QXR_5 oysters had MDS profiles that were more consistent with the wild-type population than with other QXR oysters. Again this suggests that genetically susceptible oysters remain in the QXR_5 population. Despite this, most QXR oysters clustered tightly together and there was a clear progression from wild-type, through QXR_5, to QXR_6 phenotypes. This is consistent with continuing selection of QX disease resistance. It was also evident that at least some oysters bred for combined resistance to Winter Mortality Syndrome and QX disease (WB oysters) had phenotypes that closely resembled to QXR_6 population, suggesting that different selection regimes may lead to similar genetic outcomes.

Overall the data presented here suggest that numerous genetic factors contribute to QX disease-resistance in Sydney rock oysters and that proteomic analysis is a useful way of identifying disease-resistant oysters for selective breeding.

Acknowledgments

This study was funded in part by the Australian Fisheries Research and Development Corporation (FRDC) in conjunction with the NSW Department of Primary Industries. We thank Daniel Butt for helping with sampling in Georges River.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jprot.2009.06.012.

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