Co-option of bacteriophage lysozyme genes by bivalve genomes

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Eukaryotes have occasionally acquired genetic material through horizontal gene transfer (HGT). However, little is known about the evolutionary and functional significance of such acquisitions. Lysozymes are ubiquitous enzymes that degrade bacterial cell walls. Here, we provide evidence that two subclasses of bivalves (Heterodonta and Palaeoheterodonta) acquired a lysozyme gene via HGT, building on earlier findings. Phylogenetic analyses place the bivalve lysozyme genes within the clade of bacteriophage lysozyme genes, indicating that the bivalves acquired the phage-type lysozyme genes from bacteriophages, either directly or through intermediate hosts. These bivalve lysozyme genes underwent dramatic structural changes after their co-option, including intron gain and fusion with other genes. Moreover, evidence suggests that recurrent gene duplication occurred in the bivalve lysozyme genes. Finally, we show the co-opted lysozymes exhibit a capacity for antibacterial action, potentially augmenting the immune function of related bivalves. This represents an intriguing evolutionary strategy in the eukaryote–microbe arms race, in which the genetic materials of bacteriophages are co-opted by eukaryotes, and then used by eukaryotes to combat bacteria, using a shared weapon against a common enemy.

1. Introduction

It has been well established that horizontal gene transfer (HGT), the movement of genetic materials between distinct evolutionary lineages, plays an essential role in the evolution of prokaryotic genomes [1–3]. Moreover, during the establishment of the mitochondrion and the plastid, large amounts of genetic material from the endosymbionts were transferred to the eukaryotic nuclear genomes. In contrast, the ongoing, subsequent HGT events in eukaryotes have long been underappreciated [3]. Recently, more and more cases of HGT have been reported in eukaryotes [3].

Lysozymes are ubiquitous bacteriolytic enzymes found in most life forms and viruses, hydrolysing peptidoglycans in bacterial cell walls [4–6]. Several distinct lysozyme classes have been described, including hen egg-white lysozyme (HEWL; glycoside hydrolase 22 [GH22]), goose egg-white lysozyme (GEWL or GH23), bacteriophage T4 lysozyme (T4 L or GH24) and GH25
lysozyme typically found in bacteria [5,7–9]. Different classes of lysozymes lack any obvious similarity at the sequence level, but share certain (albeit distant) similarity in their three-dimensional structures [4]. Essentially degrading bacterial cell walls, lysozymes function in various biological processes, such as defence of bacterial infections (animals and plants), digestion of bacteria as food (animals and protozoa), cell wall synthesis and remodelling (bacteria), and lysis of bacteria at the end of the phage replication cycle [8].

Recently, GH25 lysozyme was found to be repeatedly transferred from bacteria to fungi, insects, plants and archaea [10]. The horizontally transferred lysozymes are used as antibacterial molecules by recipients and may thus complement the recipients’ response to bacteria [10]. However, much remains unknown about both their evolutionary and functional implications. In a specific potential case of HGT, a phage-type-like lysozyme (GH24 lysozyme) was identified in the Manila clam (Ruditapes philippinarum) [9], but the evolutionary history and mechanism of acquisition of this lysozyme have not been well established. HGT was suggested to be ‘the most probable explanation’ [9]. However, the possibility of this lysozyme being a laboratory artefact or contaminant has not been formally excluded.

In this study, we provide evidence that phage-type GH24 lysozyme genes were horizontally transferred into bivalve genomes several hundred million years ago. The co-opted lysozyme genes have undergone dramatic gene structural changes and multiple independent gene duplication events. We also provide evidence that the co-opted phage-type lysozymes have an antibacterial function in their new hosts. This may be a general strategy for evolutionary co-option of specific weapons from the arsenal of one species using HGT, and understanding this case will help advance our understanding of the mechanisms and evolutionary pressures surrounding HGT in eukaryotes.

2. Results

2.1. Identification of phage lysozyme-like genes in the bivalve genomes

Through analysis of the transcriptome sequences of various bivalves and experimental work, we uncovered 19 sequences that share significant similarity with GH24 phage-type lysozyme in 11 bivalves (figure 1; electronic supplementary material, tables S1 and S2). The 11 bivalve species used in this study belong to two subclasses of Bivalvia: Heterodonta and Palaeoheterodonta (figure 1; electronic supplementary material, tables S1 and S2). The 11 bivalve species used in this study belong to two subclasses of Bivalvia: Heterodonta and Palaeoheterodonta (figure 1; electronic supplementary material, tables S1 and S2). The 11 bivalve species used in this study belong to two subclasses of Bivalvia: Heterodonta and Palaeoheterodonta (figure 1; electronic supplementary material, tables S1 and S2). The 11 bivalve species used in this study belong to two subclasses of Bivalvia: Heterodonta and Palaeoheterodonta (figure 1; electronic supplementary material, tables S1 and S2). The 11 bivalve species used in this study belong to two subclasses of Bivalvia: Heterodonta and Palaeoheterodonta (figure 1; electronic supplementary material, tables S1 and S2). The 11 bivalve species used in this study belong to two subclasses of Bivalvia: Heterodonta and Palaeoheterodonta (figure 1; electronic supplementary material, tables S1 and S2). The 11 bivalve species used in this study belong to two subclasses of Bivalvia: Heterodonta and Palaeoheterodonta (figure 1; electronic supplementary material, tables S1 and S2). The 11 bivalve species used in this study belong to two subclasses of Bivalvia: Heterodonta and Palaeoheterodonta (figure 1; electronic supplementary material, tables S1 and S2). The 11 bivalve species used in this study belong to two subclasses of Bivalvia: Heterodonta and Palaeoheterodonta (figure 1; electronic supplementary material, tables S1 and S2). The 11 bivalve species used in this study belong to two subclasses of Bivalvia: Heterodonta and Palaeoheterodonta (figure 1; electronic supplementary material, tables S1 and S2). The 11 bivalve species used in this study belong to two subclasses of Bivalvia: Heterodonta and Palaeoheterodonta (figure 1; electronic supplementary material, tables S1 and S2).

2.2. The origin of bivalve lysozyme genes

To investigate the origin of the phage T4 lysozyme-like sequences in the bivalve genomes, we conducted phylogenetic analyses of the lysozyme protein sequences of phages, bacteria and bivalves. We found that the bivalve lysozyme sequences form a monophyletic group with strong statistical support: Bayesian posterior probability (PP) = 0.98. The phylogenetic tree also shows that the bivalve lysozyme clade robustly nests within the clade of phage lysozymes (PP = 1.00). The closely related taxa are the phages isolated in the Mediterranean sea [11,12]. The phylogenetic pattern indicates that these bivalve lysozyme sequences ultimately originated from bacteriophages. We thus designate these bivalve sequences bivalve co-opted phage lysozyme-like (BCPL) genes.

2.3. Frequent gene structure changes after co-option

Phage lysozyme genes do not contain any introns. To our surprise, the BCPL genes contain at least one intron (figure 2). For WbLysO2, CpLysO1/2, HcLysO1/2 genes, we were able to obtain only partial gene sequences, but inferred that they contain at least one intron based on mRNA sequence information (figure 2). This represents a compelling example of intron gain in eukaryotic species. However, we do not observe any general trend for the intron gain and the evolution of intron sizes. The introns do not share any significant similarity with each other, indicating they are not under strong functional constraint.

We also observed at least three potential gene fusion events involving the BCPL genes (figure 2; electronic supplementary material, figure S2). The BCPL genes (PaLysO1, MiLysO1 and WbLysO2) were fused into novel genes with the PGRP gene, the MAM and LDL-receptor class A domain-containing protein 2-like (MALRD2) gene and the basement membrane-specific heparan sulfate proteoglycan core protein-like (HSPG) gene in Panopea abrupta, Morrella iridescens and Wenbei, respectively. All three fusion genes can be expressed as a single transcript. Interestingly, for the PGRP-PaLysO1 fusion gene, the PaLysO1 can be expressed independently (electronic supplementary material, figure S2).
Figure 1. (a) The phylogenetic tree of bacterial, phage and bivalve lysozymes. The bacterial, phage and bivalve lysozymes are highlighted in turquoise, red and blue, respectively. The topology was inferred using a Bayesian methodology. The numbers on the selected nodes represent Bayesian posterior probabilities. (b) The bivalve phylogeny is based on the one inferred using the cox1 gene (electronic supplementary material, figure S1). Bivalve abbreviations: Sp, Saxidomus purpuratus; Mm, Meretrix meretrix; Rp, Ruditapes philippinarum; Mi, Moerella iridescens; Pa, Panopea abrupta; Ss, Solen strictus; Wb, Wenbei; Qcb, Qicaibei; Hc, Hyriopsis cumingii; Cp, Cristaria plicata; Aw, Anodonta woodiana.
2.4. Recurrent gene duplication of bivalve lysozyme genes

Our phylogenetic analysis shows that at least four independent gene duplication events occurred within the BCPL genes: (i) one in the common ancestor of *Ruditapes philippinarum*, *Meretrix meretrix*, *Saxidomus purpuratus*, Qicaibei and Wenbei; (ii) two in the common ancestor of *Hyriopsis cumingii*, *Cristaria plicata* and *Anodonta woodiana*; and (iii) one in the lineage leading to *S. strictus*. The absence of related lysozyme gene copies (figure 1), namely *SpLyso2*, *AwLyso2*, *AwLyso3* and *HcLyso3*, might be because of either gene loss or failure of transcriptome sequence due to the specific expression patterns of related genes. Either possibility does not change our conclusions regarding the recurrent nature of these duplication events.

2.5. Antibacterial activity of bivalve co-opted phage lysozyme-like genes

Because the BCPL proteins are derived from phage-type lysozymes, we hypothesized that the BCPL proteins might possess antibacterial capacity in bivalves. To test this hypothesis, we focused on the BCPL genes of *H. cumingii*. *H. cumingii* genome encodes at least two copies of BCPL genes (i.e. *HcLyso1* and *HcLyso2*). We first investigated the expression pattern of the BCPL genes of *H. cumingii*. We found evidence that both *HcLyso1* and *HcLyso2* can be expressed in haemocytes, hepatopancreas, gills and mantle (figure 3a,b). However, the *HcLyso1* and *HcLyso2* genes have both overlapping and divergent expression patterns: *HcLyso1* has an elevated expression level in hepatopancreas and gills, whereas *HcLyso2* has an elevated expression level in hepatopancreas and mantle. The expression difference indicates a
potential functional divergence between HcLyso1 and HcLyso2 genes. Nevertheless, these results suggested that both H. cumin-
gii BCPL genes are functionally active. Next, we used Vibrio
parahemolyticus and Bacillus cereus to challenge H. cumingii.
After both bacterial challenges, the HcLyso1 gene expression
level first decreased and then increased, but the HcLyso2 gene
expression level significantly increased (figure 4
a–f). Finally,
we cloned, expressed and purified the HcLyso1 protein and
measured its bacteriolytic activity. Our results suggest that
the purified HcLyso1 protein exhibits the capacity to degrade
bacteria (figure 3
c,d). The optimal reaction temperature and
pH are approximately 30°C and approximately 10.0, respect-
ively (figure 3
c,d). Hyriopsis cumingii is widely distributed
within the temperature zone across China. The growth rate
is highest from June to October, when the average water
temperature is 20–31°C [13]. This fact is compatible with
our experimental result, and as bacterial growth rates are
typically higher in warmer water, the optimal reaction tem-
perature may reflect this. As for the optimal pH, we suspect it
might be related to the specific tissue/cellular environment;
however, the possibility that in vitro experiments do not
reflect the actual lysozyme activity cannot be formally
excluded. Taken together, these lines of evidence indicate
that the H. cumingii BCPL genes respond to bacterial
challenges and function in antibacterial activity. It follows
that the co- opted lysozymes may augment the immune func-
tion of bivalves by actively degrading bacterial cell walls.

3. Discussion

HGT, the acquisition of genetic materials from distinct
evolutionary lineages, is thought to have played some role
in the evolution of eukaryotes. In this study, we report
that lysozyme genes previously described in bivalves
[9] were horizontally transferred from bacteriophages to
two subclasses of bivalves. Subsequently, these horizontally
acquired genes underwent both dramatic structural changes
in the bivalve genomes—including intron gain and fusion
with other genes—as well as recurrent gene duplication.
Functional analyses show that these bivalve lysozymes can
degrade bacterial cell walls. Therefore, the horizontally
acquired lysozyme genes are likely to be acting to augment
bivalves’ capacity to restrict bacterial infections. We believe
this type of evolutionary strategy for host–microbe inter-
action might be prevalent over evolutionary time. While
phage-type-like lysozyme has been identified in the Manila
clam (R. philippinarum) [9], that work proposed the
occurrence of HGT only as a probable explanation, and did not explore the evolutionary history and mechanism of this lysozyme in details. In this study, we have not only provided evidence that the bivalve genomes acquired phage-type lysozyme genes via HGT, but also explored the detailed evolutionary history and functional consequence of the co-option of a phage lysozyme by bivalves.

Our phylogenetic analyses suggest that the ultimate donors are closely related to the phages isolated in the Mediterranean sea. It seems that the donors and recipients coexisted in the same ecological niche (a water environment), providing physical opportunity for HGT to occur. For the origin of the BCPL genes, we propose two competing evolutionary scenarios: (i) direct transfer (an HGT event took place from bacteriophages to bivalves directly); or (ii) transfer via intermediate (the HGT was mediated by bacteria, i.e. bacteriophage to bacteria and then to bivalve). However, the transfer via intermediate scenario requires at least two independent transfer events (bacteriophages to bacteria and bacteria to bivalve), which is less parsimonious than the direct transfer scenario. It is also worth noting that we observed no bacterial sequences within our sequenced genes of interest or the flanking regions we examined, we observed no bacterial sequences within our sequenced genes. It is also worth noting that the ultimate donors and recipients coexisted in the same ecological niche (a water environment), providing physical opportunity for HGT to occur. For the origin of the BCPL genes, we propose two competing evolutionary scenarios: (i) direct transfer (an HGT event took place from bacteriophages to bivalves directly); or (ii) transfer via intermediate (the HGT was mediated by bacteria, i.e. bacteriophage to bacteria and then to bivalve).

While HGT is likely to be random, persistence of horizontally transferred elements would be expected either when they are source is likely to be low and would be less parsimonious. A recent phylogenomic study reveals the Bivalvia class constitutes a monophyletic group [14]. However, the relationship among the four subclasses of the Bivalvia (Heterodonta, Palaeoheterodonta, Pteriomorphia and Protobranchia) is still controversial [14–17]. If these BCPL genes originated from a single HGT event, then it took place before the divergence of Heterodonta and Palaeoheterodonta, but after the divergence of Pteriomorphia and Heterocancha, given no BCPL gene was identified in the complete genomic sequence of C. gigas (subclass Pteriomorphia). The origin of Heterodonta is estimated to be approximately 490 million years ago (Ma) [15]. It follows that the most recent common ancestor of Heterodonta and Palaeoheterodonta should be older than 490 Ma, but not older than 520–530 Ma, when Bivalvia arose [15]. This time scale should be taken with caution, as the time scales and the phylogenetic relationships of bivalve species are still controversial [14–17]; however, the conclusion remains that a single event is more likely given our results.

As a potential strategy for dealing with bacteria, exaptation of bactericidal enzymes would represent a potentially fruitful strategy for any eukaryote. Presumably, phage lysozymes have adapted to prey on bacteria long before the evolution of multicellular organisms, and therefore they have been uniquely shaped by evolutionary pressure to affect bacterial cell walls. HGT would allow the eukaryote to ‘short cut’ the slow evolutionary process of adapting an endogenous protein of bactericidal enzymes would represent a potentially fruitful strategy for any eukaryote. Presumably, phage lysozymes have adapted to prey on bacteria long before the evolution of multicellular organisms, and therefore they have been uniquely shaped by evolutionary pressure to affect bacterial cell walls. HGT would allow the eukaryote to ‘short cut’ the slow evolutionary process of adapting an endogenous protein to deal with bacterial infection. However, co-option is of course not without risks to the organism, such as disrupting genomic features and the cellular networks of recipients [18]. While HGT is likely to be random, persistence of horizontally transferred elements would be expected either when they are

Figure 4. The expression change of the H. cumingii BCPL genes after bacterial challenges. (a,d) Vibrio parahemolyticus and (b,e) B. cereus were used to challenge H. cumingii. (c,f) PBS treatment was used as a control. The relative expression levels of (a–c) HcLyso1 and (d–f) HcLyso2 genes were examined in the gills of H. cumingii. The difference of gene expression between hour 0 and other time points was analysed using an unpaired sample t-test. The significance levels of change were indicated by an asterisk (0.01 < p < 0.05) or two asterisks (p < 0.01). Error bars indicate standard deviation of the mean.
4. Material and methods

4.1. Samples and transcriptome sequencing

The bivalve samples (electronic supplementary material, table S1) were purchased in local markets in Nanjing (Jiangsu Province), Hangzhou (Zhejiang Province) and Wuhu (Anhui Province). The species were identified based on morphological traits and have been confirmed by sequencing the mitochondrial cytochrome c oxidase subunit I (cox1). Throughout this study, RNA was extracted using the RNApure High-purity Total RNA Rapid Extraction kit (spin-column; Bioteke, Beijing, China). The transcriptomes of the 11 bivalve species were sequenced, using an Illumina HiSeqTM 2000. The sequencing reads were de novo assembled, using the TRINITY program. We searched for phage-type lysozyme protein homologues (cut-off E-value of 10^-05 with >100 alignable residues), using the BLAST algorithm against the NCBI non-redundant protein sequence database. Representative lysozyme sequences were chosen for phylogenetic analysis. Protein sequences were aligned, using the MAFFT multiple sequence alignment program [21] and then manually edited. The phylogenetic tree was inferred with a Bayesian method available in MrBayes v. 3.1.2 [22]. The best-fit substitution model was selected based on PROTEST v. 3 [23], and the BLOSUM62 + I + G model was employed. A total of 5,000,000 generations in four chains were run, sampling posterior trees every 100 generations. The first 25% of the posterior trees were discarded. The phylogenetic trees were viewed using FigTree v. 1.4.2.

4.2. Cloning the full-length BCPL cDNA

To obtain the full-length of the BCPL cDNA, we performed both 5'- and 3'-rapid amplification of cDNA ends (RACE), using SMARTer RACE 5'/3' Kit (Clontech), following the manufacturer's manual. All the primers used in RACE are listed in electronic supplementary material, table S3. The RACE PCR products were sequenced by Springen (Nanjing, China). The full-length HcLyso2 and CplLyso1 cDNA sequences were obtained by performing RACE with primers designed based on CplLyso2 (CplLyso1-F and CplLyso1-R) in electronic supplementary material, table S3) and HcLyso1 (HcLyso2-F and HcLyso2-R in electronic supplementary material, table S3) genes, respectively.

4.3. Genome walking in the BCPL genes

Genomic DNA was extracted using NucleoSpin Tissue (Clontech). To obtain the flanking genomic regions of the BCPL genes, genome walking was carried out using a Universal GenomeWalker 2.0 kit (Clontech) according to the manufacturer's manual. In brief, genomic DNA was digested by DnlI, EcoRV, PstI and SmaI, separately. The digested genomic DNA was purified using NucleoSpin Gel and PCR Clean-Up kit (Clontech). Each batch of the purified genomic DNA was ligated to the GenomeWalker adaptor. Nested PCRs were then conducted. All the primers used in genome walking were listed in electronic supplementary material, table S4. The resulting PCR amplicons were sequenced by Springen (Nanjing, China). The sequences reported in this paper have been deposited in the GenBank database (accession nos. KT934018–KT934048).

4.4. Phylogenetic analysis of lysozymes

The BLAST algorithm was employed to search against the NCBI non-redundant protein sequence database for the homologues of BCPL proteins. Representative lysozyme sequences were chosen for phylogenetic analysis. Protein sequences were aligned, using the MAFFT multiple sequence alignment program [21] and then manually edited. The phylogenetic tree was inferred with a Bayesian method available in MrBayes v. 3.1.2 [22]. The best-fit substitution model was selected based on PROTEST v. 3 [23], and the BLOSUM62 + I + G model was employed. A total of 5,000,000 generations in four chains were run, sampling posterior trees every 100 generations. The first 25% of the posterior trees were discarded. The phylogenetic trees were viewed using FigTree v. 1.4.2.

4.5. Expression of fusion genes

The expression of fusion genes, PGRP-PaLyso1 of P. abrupta, MLRD2-MiLyso1 of M. iridescens, and HSPG-WbLyso2 of Wenbei, were carried out using 5'-RACE cDNA with the mixture of UPM-long and UPM-short primers and lysozyme gene-specific primers listed in electronic supplementary material, table S5.

4.6. Expression pattern of HcLyso1 and HcLyso2

Haemocytes, hepatopancreas, gills and mantle were sampled from healthy H. cumingii for RNA isolation. Diluted V. Parahaemolyticus and B. cereus cultures (approx. 3 x 10^7 cells) were injected into the adductor muscles of H. cumingii. As a control, H. cumingii was also treated with the phosphate-buffered saline (PBS) solution. At 0, 2, 6, 12 and 24 h post-bacterial challenge or PBS treatment, the gills from three H. cumingii were sampled for RNA extraction. RNA was reverse transcribed with PrimeScript RT Master Mix (Perfect Real Time) for qRT-PCR analysis. For qRT-PCR analysis, we used SYBR Premix Ex Taq II (Tli RNaseH Plus; Dalian, China) and the actin gene as a reference. All the samples were repeated in triplicate. The 2^-ΔΔCt method was used to analyse the relative changes in gene expression [24]. The difference of gene expression between hour 0 and other time points was analysed using an unpaired-sample t-test.

4.7. Bacteriolytic activity of HcLyso1 protein

In-Fusion PCR Cloning kit (Clontech) was used for the construction of recombinant vector. The HcLyso1 gene (the
whole open reading frame, 456 bp) was obtained using gene-specific primers with 16 bp vector sequences at the 5’ end and Clontech SeqAmp DNA polymerase. The amplified gene fragment was then cloned into the pET30a vector linearized by EcoRI and XhoI. The recombinant vector was transformed into Trans-T1 cells, and the positive clone was sequenced to ensure correct insertion. The recombinant vector was then transformed into BL21(DE3) cells for protein expression. After denaturation and renaturation as described previously [25], the recombinant protein with His-tag was purified using His-Bind resin chromatography (Novagen).

The bacteriolytic activity of HcLyso1 at different temperature or pH levels was analysed using lysozyme assay kit (Jiancheng, Nanjing, China). In principle, lysozyme activity is measured by tracking the increase in transmittance as the enzyme degrades the bacterial cell wall. A total of three mixtures were prepared: blank mixture (0.2 ml distilled water, 2.0 ml working solution Micrococcus luteus), standard mixture (0.2 ml of standard lysozyme solution, 2.0 ml working solution M. luteus) and sample mixture (0.2 ml HcLyso1 solution, 2.0 ml working solution M. luteus). The bacteriolytic activity of HcLyso1 can be calculated through the following formula:

\[
\text{activity of HcLyso1} = \frac{T_{15} - B_{15}}{S_{15} - B_{15}} \times \text{activity of standard} \times \text{dilution factor},
\]

where \(T_{15}\) represents the transmittance at 530 nm after the sample mixture was treated under different temperature or pH levels for 15 min; \(B_{15}\) represents the transmittance at 530 nm after the blank mixture was placed in 37°C for 15 min; \(S_{15}\) represents the transmittance at 530 nm after standard mixture was placed in 37°C for 15 min; and the activity of standard is 200 U ml\(^{-1}\).

Data accessibility. The sequences reported in this paper have been deposited in the GenBank database (accession nos KT934018–KT934048). Any constructs created are available upon request. The author responsible for distribution of materials integral to the findings presented in this article is G.-Z.H. (guanzhu@njnu.edu.cn).

Authors’ contributions. G.-Z.H., Q.R. and W.W. designed the study. Q.R., K.H., J.T. and Z.W. performed the molecular experiments. C.W. and G.-Z.H. performed the evolutionary analyses. M.J., J.L. and T.Y. contributed reagents/materials. G.-Z.H. and Q.R. analysed the data. G.-Z.H. and G.J.W. wrote the manuscript with inputs from Q.R.

Competing interests. We declare we have no competing interests.

Funding. This study was supported by the National Natural Science Foundation of China (grant no. 31572264), the Natural Science Foundation of Jiangsu Province (BK20131401, BK20161016), the Natural Science Fund of Colleges and universities in Jiangsu Province (13KJB240002, 14KJA240002), the High Level Talents Foundation in Nanjing Normal University (2012104XQG0101) and the Priority Academic Programme Development (PAPD) of Jiangsu Higher Education Institutions.

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