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Full Length Research Paper

Suppression-subtractive hybridization: A rapid and inexpensive detection methodology for up-regulated *Perkinsus olseni* genes

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A cDNA forward subtraction library was constructed from the mollusc parasite *Perkinsus olseni* exposed to hemolymph from its natural host, the clam *Ruditapes decussatus,* and two different methodologies were used to unravel different non-redundant contigs. Our results demonstrated that screening of the non-enriched direct cDNA subtractive library (Dfsl) was the most efficient and least time- consuming method. It facilitated the identification of genes belonging to 25 different classes of molecular functions out of the 96 clones analyzed. In contrast, only 6 different classes from 204 sequenced clones were identified from the enriched library (efMOSl). It was concluded that the Dfsl cDNA subtractive library resulted in a larger pool of diversified gene hits that were obtained in a shorter time and with less technically-demanding methodology when compared to the efMOSl approach, thus demonstrating its significance and usefulness when time and/or resources are limited.

Key words: Unicellular parasite; *Perkinsus olseni*; SSH; bacterial dot blot and plate lifts.

INTRODUCTION

Highly efficient methods for gene identification are required to perform genome annotations and molecular characterization of microbiological systems not yet fully characterized, like the unicellular mollusc parasite *Perkinsus olseni* (Lester and Davis 1981) exposed to hemolymph from its natural host, the clam *Ruditapes (Tapes) decussatus* (Linnaeus, 1758). Several strategies are available for the analysis of differential gene expression on a whole genome scale, each of them claiming specific advantages. However, and although less discussed, they also have intrinsic disadvantages. Differential display approaches (Liang et al., 1994) are suitable not only for the less complex genomes but also for those with a higher degree of complexity. Libraries constructed through suppression-subtractive hybridization (SSH), followed by colony blot hybridization or cDNA amplified fragment length polymorphism (AFLP), are time-consuming because positive results have to be confirmed by an independent technique (Massart and Jijakli, 2006).

The combination of SSH and negative subtraction chain (NSC) ensures that target sequences are dramatically and efficiently enriched, but may not have enough sensitivity (Li et al., 2005) to directly isolate differentially expressed genes from highly complex eukaryote genomes (Lisitsyn et al., 1993). Microarray techniques, on the other hand, are expensive and their use is frequently limited to the most standardized models (Heller, 2002).

In the SSH method, subtraction and normalization are performed simultaneously (Mahalingam et al., 2003) thus equalizing the abundance of target cDNAs in the subtracted population (Diatchenko et al., 1996). To decrease the number of background clones in the libraries generated by SSH, a mirror orientation selection procedure (MOS) (Rebrikov et al., 2000) was developed but “false positive” clones were not entirely eliminated due to the simultaneous use of polymerase chain reaction amplification. In the present study, our main objective was the characterization of the gene ontology and molecular function in order to compare results obtained by two different SSH approaches: (1) the direct forward subtractive library (Dfsl), which consists on direct cloning of the forward cDNA subtractive library, followed by plate lifts screening to reveal up-regulated genes, and (2) the MOS enrichment methodology, used after completion of the SSH step and followed by *in situ* differential screening by bac-

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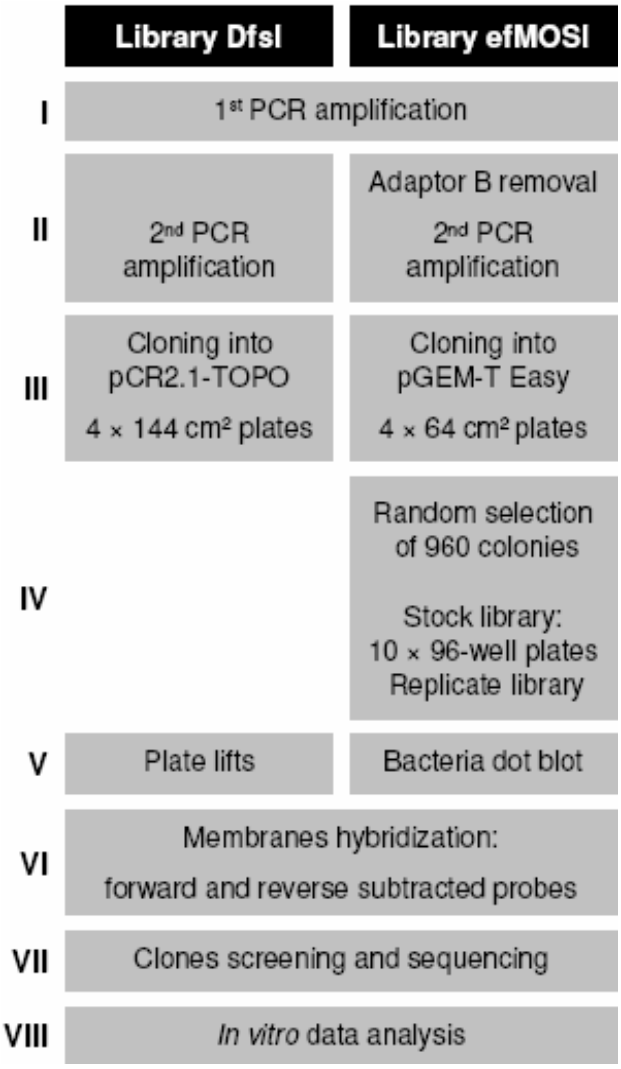


Figure 1. Comparison of the two methodologies followed for construction of the subtracted libraries, represented in eight stages (Diatchenko et al.,1996) and (Rebrikov et al., 2000). DfsI, Direct forward subtractive library and efMOSI, enriched forward Mirror Orientation Selection library.

teria dot blot (efMOSI). We conclude that the use of direct cloning and plate lift screening is a simple, inexpensive and efficient method for identifying parasite gene diversity.

MATERIALS AND METHODS

P. olseni clonal culture

The clonal culture of *P. olseni* was maintained in DME: Hams F12 (1:2) medium (Gibco, Grand Island, NY, USA) supplemented with 5% FBS (fetal bovine serum, Invitrogen, Carlsbad, USA) according to the modified method (Robledo et al., 2002) of Gauthier and Vasta (1995). Prior to treatment, cultures were maintained for 3 days at 28ºC in exponential growth phase with no medium changes A two-day parasite cell culture was exposed to *R. decussatus* fresh collected hemolymph and this population was named "*tester*" while a control parasite cell culture population, not challenged with hemolymph, was designated "*driver*".

RNA preparation

Total RNA was extracted using the RNeasy total RNA isolation Kit (Qiagen, Chatworth, USA), according to the manufacturer's procedures, after mechanical disruption of cells to increase cell lysis efficiency. Following spectrophotometric quantification, the integrity of the RNA was confirmed by gel electrophoresis. For SSH library construction, mRNA was purified from total RNA isolated from both cell populations (tester and driver) using the Oligotex mRNA Midi Kit (Qiagen), according to manufacturer's instructions.

Construction of the direct cDNA subtractive library (DfsI)

To target up-regulated genes, *P. olseni* SSH cDNA library was constructed by subtracting the *tester* from *driver* mRNA. For the SSH library, the forward and reverse subtractive libraries were constructed using the PCR-SELECT cDNA Subtraction Kit (Clontech, Palo Alto, USA) according to manufacturer's instructions, and PCR optimized to 10 cycles. Ten microliters of forward subtractive library PCR mixture was then submitted to dATP addition by performing a 15 min PCR step with DNA polymerase (Invitrogen), in order to improve T/A ligation efficiency into TOPO cloning vector (Invitrogen). The forward subtractive library, consisting of cDNA fragments ligated into TOPO pCR II, was used to transform DH5α cells (Invitrogen) which were subsequently grown in agar plates (144 cm^2) with 100 µl X-gal (20 mg/ml) and 5 µl IPTG (100 mg/ml). To perform the screening, the library was transferred to Hybond^{TM}-XLfilters (Amersham Biosciences, Piscataway, USA) by bacterial plate lifts (Sambrook et al., 1989). Duplicate filters were prepared for each master plate.

Construction of the MOS enriched library (efMOSI)

Mirror oriented selection (MOS) technique was performed in order to enrich the library with differentially expressed genes (Rebrikov et al., 2000). Briefly, PCR product was purified using the GFX column PCR DNA clean (Amersham Biosciences) and re-suspended in TE buffer (pH 8) up to a concentration of 20 – 30 ng/µl. 5 µl of this purified product was XmaI- digested (New England Biolabs, Ipswich, USA) to remove the NP1 adaptor (B adaptor, Figure 1) and 1 µl of the 15 µl of digestion inactivated product was denatured for 1.5 min at 98ºC and hybridized for 12 h at 68ºC. One microliter of a 40x dilution of hybridization product was used for PCR amplification and 1µl of the resulting product was inserted into the T/A cloning vector pGEM®-T Easy (Promega, Madison, USA). Individual transformants of DH5α cells, carrying exogenous cDNA fragments, were isolated from selected white colonies on X-gal/IPTG agar plates, as previously described, being stored arrayed in 10 plates of 96-wells. For screening, the 96-well plate MOS library clones grown in liquid LB were spotted (3 µl per spot) in duplicate membranes and processed by bacterial dot blot as described (Fonseca et al., 2005).

Identification of differentially up-regulated genes

Differential screening was performed by double filter hybridization. After digestion and neutralization *in situ*, each membrane was hybridized with a probe consisting on the pool of cDNAs (that is, the products of the second PCR) obtained in each subtractive library (forward or reverse). These complex probes were carefully synthesized by PCR. Briefly, four tubes of either forward or reverse subtra-

Table 1. GO analysis according to category of molecular function determined using the GOblet server.

Library	DfsI	efMOSI
Sequenced cDNA	96	204
GOblet submitted	96	108
GO subclasses	25	6
GO molecular function hit	44	21
No GO match	52	87

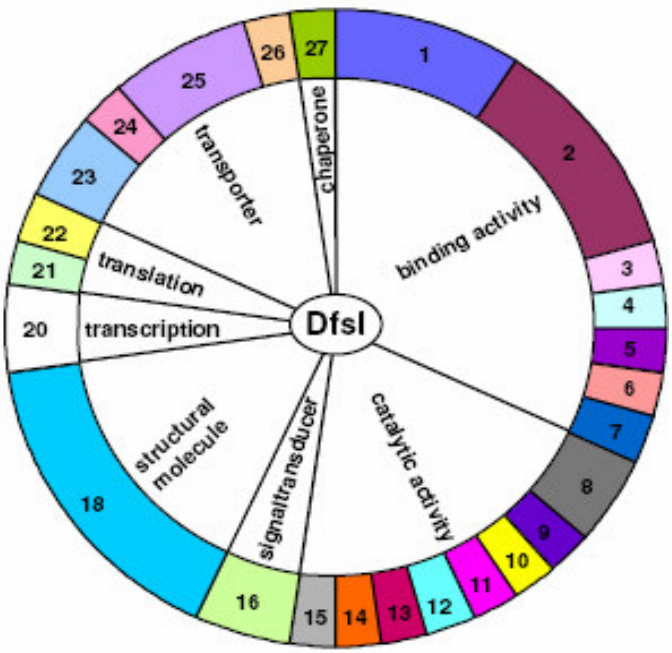
cted secondary PCRs were combined and purified using GFX column PCR DNA clean (Amersham Biosciences) according to the manufacturer's instructions, reducing the volume to 24 µl in order to concentrate the probes to 50 ng each. Afterwards, removal of the adaptors was performed by first digesting with RsaI (10U) and EaeI (10U) for 2 h at 37ºC and secondly with SmaI (10U) for 2 h at room temperature (all enzymes were from New England Biolabs). Separation was achieved by electrophoresis and DNA was recovered from the gel band using Gel Band Purification KIT (Amersham Biosciences). Adaptor-free cDNAs from forward and reverse subtractions were radio-labelled with α-^{32}P dCTP using the random prime labelling kit (Rediprime™ II, Amersham Biosciences) and purified by spin filtration (Microspin™ S.200 HK columns, Amersham Biosciences), according to manufacturer's instructions. Pre-hybridization and hybridization using UltraHyb solution (Ambion, Austin, USA) were carried out at 42ºC. Membranes were then washed twice in 1x SSC, 0.1% SDS at 42ºC for 5 min, followed by two 10 min washes in a more stringent solution (0.1x SSC, 0.1% SDS) at 42ºC. Autoradiography was performed with Kodak BioMax MS film. The impressed signals were quantified by densitometry (Quantity One, BioRad, Richmond, USA). Naked eye analysis was also performed to confirm information provided by the software. DNA fragments in dots showing more than two fold differences in impressed signal strength were further processed for sequence analysis. 96 and 204 clones were analyzed from DfsI and efMOSI respectively.

In silico data treatment for gene characterization

Gene ontology annotation of the efMOSI library clones was performed after removal of vector sequence and correction of obvious reading errors, using VecScreen (www.ncbi.nlm.nih.gov), through GOblet server (http://goblet.molgen.mpg.de). On the other hand, the DfsI EST sequences were trimmed against the vector and adaptors using the contig program included in Vector NTI (Invitrogen), and submitted to GOblet . Sequence characterization used a format based on server instructions (Groth et al., 2004), defining E value <1x10^{-1} as cut-off value. The information selected was the GO hits in the molecular function category.

RESULTS AND DISCUSSION

Various approaches for SSH-based library screening have been published in the last decade. Herein, two different SSH-based approaches (efMOSI and DfsI) were tested in parallel to determine which one proved to be (i) more efficient, (ii) time-saving and (iii) less redundant for identification of differentially expressed genes from the parasite *P. olseni*, in response to the presence of hemolymph from its natural host, the carpet shell clam *R. decussatus*. Since the genome of *Perkinsus* is not yet



1 DNA binding (9,1%)
2 RNA binding (11,4%)
3 transcription factor binding (2,3%)
4 transition ion binding (2,3%)
5 purine nucleotide binding (2,3%)
6 cytoskeletal protein binding (2,3%)
7 androgen binding (2,3%)
8 ATP-dependent helicase activity (4,5%)
9 carbon-carbon lyase (2,3%)
10 oxidoreductase (2,3%)
11 hydrolase on carbon-nitrogen bond (2,3%)
12 peptidase activity (2,3%)
13 forming carbon-nitrogen bonds (2,3%)
14 transferring acyl groups (2,3%)
15 transferring one-carbon group (2,3%)
16 transmembrane receptor (4,5%)
18 ribosome structural molecule (15,9%)
20 transcription factor activity (4,5%)
21 translation initiation factor (2,3%)
22 translation termination factor (2,3%)
23 amino acid transporter (4,5%)
24 electrochemical transport (2,3%)
25 electron transporter (6,8%)
26 cation transporter activity (2,3%)
27 heat-shock protein activity (2,3%)

Figure 2. Results of DfsI GO analysis. The classes and subclasses are represented according to the molecular function category of GOblet tool and the area is proportional to EST sequence number. DfsI, Direct forward subtractive library.

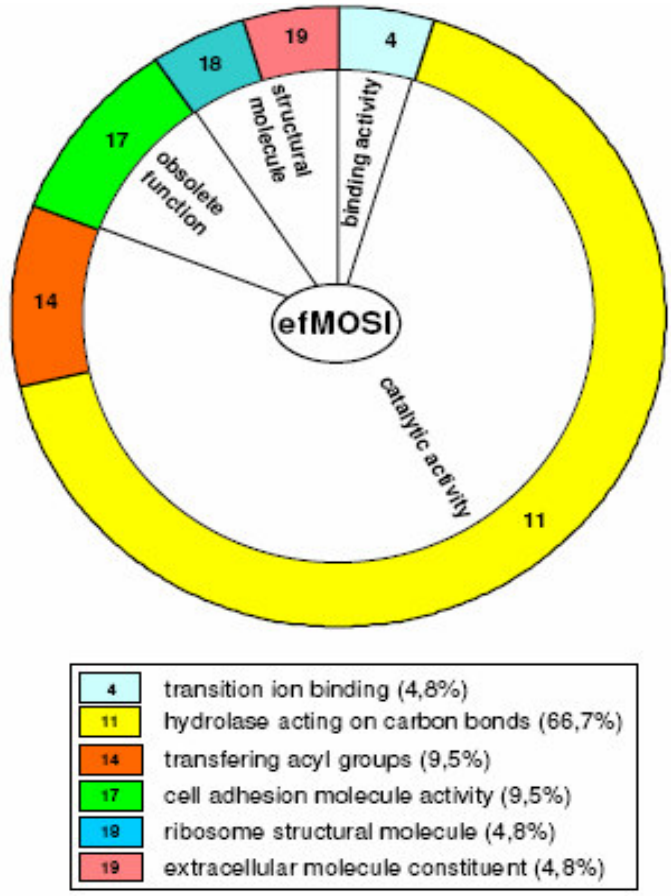


Figure 3. Results of efMOSI GO analysis. The classes and subclasses are represented according to the molecular function category of GOblet tool and the area is proportional to EST sequence number. efMOSI, enriched forward Mirror Orientation Selection library.

well characterized, the Goblet routine, consisting of a gene ontology (GO) search server based on similarity searches against known protein databases (Hennig et al., 2003; Groth et al., 2004), was chosen to annotate the anonymous sequences. GO had previously been demonstrated to be very efficient for the identification of transcriptional signatures conserved among species (McCarrol et al., 2004). The main goal of the present work was therefore to determine which screening methodology allowed identification of a larger diversity of GO classes with less time and effort.

DfsI was found to be less labour-intensive than efMOSI since step IV, which involved the manipulation of 960 clones twice before spotting, was omitted (Figure 1). Another disadvantage noted for efMOSI was the independent dot blotting of bacterial clones into two different membranes. This procedure may introduce experimental and/or interpretational errors because hybridizations have to be performed in two separate blotting experiments. In contrast, the DfsI procedure utilises the same colonies that are Present in both replica filters. Through the plate lifts and filter hybridization, extra sensitivity compensates for small differences in the amount of bacteria attached to each replica filter. Also, the screening procedure was simpler for eight DfsI membranes than for twenty efMOSI bacteria dot blots (Figure 1).

Screening analysis was also more efficient for DfsI since 100% of the 96 clones selected resulted in useful sequences. In contrast, out of the 204 clones selected from efMOSI, only 108 (53%) resulted in quality sequences (Table 1). Furthermore, *in silico* analysis of the efMOSI-derived cDNA sequences required individual cleaning from pGEM®T-easy vector sequence contamination, prior to storage in local databases. In contrast, all DfsI-derived cDNA sequences were trimmed together in one single bioinformatics step, a lesser labour-intensive procedure.

The diversity of molecular functions obtained was also significantly higher in the DfsI approach when compared to efMOSI: from 108 clones screened by *in situ* differential screening, only 6 different GO function classes were identified, whereas analysis of 96 clones obtained from plate lift screening resulted in 25 different GO classes (Table 1). The most represented molecular function class in DfsI was binding activity (32% with 14 out of 44 different ESTs obtained). From these 14 clones, 7 represented different subclass hits, indicating a higher diversity (Figure 2) than with efMOSI where, for example, within the GO class of catalytic activity, the resulting positive 16 ESTs fell into only two sub-classes (Figure 3).

The diversity was then confirmed by individual BLASTx analysis, resulting in 27 singular hits out of the 108 tested from efMOSI, while 75 different hits were obtained from DfsI (our unpublished data). In conclusion, DfsI cDNA subtractive library resulted in a larger pool of diversified gene hits, obtained in a shorter time and with less technically-demanding methodology when compared to the efMOSI approach, thus emphasizing its usefulness when limited time and/or resources are available.

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