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MINI-REVIEW

Suppression Subtractive Hybridization (SSH) and its modifications in microbiological research

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Abstract Suppression subtractive hybridization (SSH) is an effective approach to identify the genes that vary in expression levels during different biological processes. It is often used in higher eukaryotes to study the molecular regulation in complex pathogenic progress, such as tumorigenesis and other chronic multigene-associated diseases. Because microbes have relatively smaller genomes compared with eukaryotes, aside from the analysis at the mRNA level, SSH as well as its modifications have been further employed to isolate specific chromosomal locus, study genomic diversity related with exceptional bacterial secondary metabolisms or genes with special microbial function. This review introduces the SSH and its associated methods and focus on their applications to detect specific functional genes or DNA markers in microorganisms.

Keywords SSH · mRNA · Genome · Modified method · Microbiological research

Introduction

In organisms, processes such as cellular growth, organogenesis and disease progression are mediated by programs of differential gene expressions. Fifteen percent of genes in higher eukaryotes, which are approximately 15,000 mRNA among 100,000 genes in the whole genome, are reported to express at only a certain physiological stage (Wang and Lu 1998). When alterations happen in the expression patterns, they can subsequently determine the cellular new fate related to biological or pathological process. Thus, identification of differentially expressed genes can lead to greater insights into the molecular mechanism underlying diseases or other biological progressions. To isolate those target genes with differentially expression patterns, a variety of methods, such as subtractive hybridization (Lamer and Palmer 1984), mRNA differentially display polymerase chain reaction (DD PCR; Liang and Pardee 1992; Liang et al. 1992) representational difference analysis (RDA; Nikolai et al. 1993; Hubank and Schatz 1994), microarray (Chee et al. 1996; Chu et al. 1998) and suppression subtractive hybridization (SSH; Diatchenko et al. 1996), have been developed. Until now, so many methods are available; each one has its advantages and disadvantages (Table 1). Generally speaking, most of the above methods were initially very labor intensive. Commercial gene chip, being an automated high throughput method, can provide quantities of information on differentially expressed genes through relatively simple procedures. However, microarray technology and its associated equipment are very expensive and beyond the reach of many academic laboratories. Furthermore, the lack of genomic sequences to serve templates for probe design restricts their use to some certain organisms although few reports have described that a variational technology (shotgun microarrays) was employed to study the expressional changes during the parasite life cycle of *Plasmodium* (Hayward et al. 2000). As a result, alternative methods are required to identify novel genes or to study nonmodel organisms, such as varieties of microorganisms.

SSH was firstly developed in Clontech's laboratories to find out the genes that are differentially expressed in two samples at the mRNA level in eukaryotes (Diatchenko et al. 1996). Compared with microarray, this method can be performed in the absence of sequence information, and

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Table 1 Comparison of advantage and disadvantage of gene expression profile

Techniques	Advantage	Disadvantage
SSH	1. Relatively smaller quantities of samples; 2. High efficacy, especially efficient for obtaining low abundance genes; 3. High specificity	1. Generally, only two samples can be compared in one SSH; 2. The results too depend on the efficacy of ligating adaptors
Microarray	1. Smallest quantities of samples; 2. Highest efficacy; 3. High specificity; 4. More than two samples can be analyzed in one experiment	1. In general, it require some sequence information in advance; 2. Low abundance genes are difficult to detect; 3. High cost
RDA	1. High specificity and low percentage of false positive; 2. cDNA RDA can be used in mRNA without polyA	1. Low percentage of obtaining the low abundance genes; 2. Repeated procedures of enzyme cut and ligation
DD PCR	1. Relatively smaller quantities of samples; 2. High efficacy, and normally low abundance genes can also be obtained; 3. More than two samples can be analyzed in one experiment	1. High percentage of false positive; 2. The amplified products are usually in the 3'-terminal region
SH	1. Low cost; 2. Relatively longer genes can be obtained in resultant library	1. Low sensitivity; 2. Largest quantities of samples 3. Labor intensive

meet the requirements for finding novel genes in the nonmodel organisms such as specific microbes and agricultural crops. In principle, SSH is based on suppression PCR, which means using the long inverted terminal repeats (LITR) to link to the ends of the tester sample to form stable panhandle-like loop structures in each denaturation–annealing cycle. Consequently, no exponential amplification occurs in nontarget sequences. Combining normalization and suppression PCR steps in a single cycle, SSH makes it possible to equalize abundance of target cDNAs in the subtracted population, and as a result, rare differentially expressed transcripts have been reported to be enriched by 1,000-fold to 5,000-fold (Diatchenko et al. 1996). Because of its high level enrichment, low background, and normalized abundance of cDNAs, SSH has been extensively used by researchers to identify differentially expressed genes that contribute to the molecular regulation of biological or pathological processes in the past decade. During this period of time, some modifications were also introduced to make it possible to directly analyze DNA at the genome level (Akopyants et al. 1998). Furthermore, some commercial companies (e.g. Clontech, Evrogen) now supply the complete SSH procedure for detecting either differentially expressed cDNAs or genomic sequences. These developments are making SSH a potentially, widely applicable method in microbiology.

Procedure of SSH and its characteristics

As we mentioned above, SSH is an efficient PCR-based subtractive hybridization method for detecting the differentially expressed genes at the mRNA level. Diatchenko et al. (1996) have described the procedure of SSH in details, and this method is generally divided into six steps, including: (1) synthesis of tester/driver cDNAs; (2) digestion by a four-base cutting restrictive enzyme; (3) separation of the tester cDNA into two samples, followed by the step of two different suppression–adapter ligations; (4) two successive subtractive hybridization; (5) PCR amplification of target sequences; and (6) construction of the subtracted library. The schematic representation of SSH is shown in Fig. 1. It should be emphasized that two sequential hybridizations are typically performed in each procedure of SSH to guarantee the enrichment and normalization of target fragments with differential expressions. In the first hybridization step, two tester samples linked with adapters 1 and 2R were mixed with a large excess of drivers and denatured separately. They are then subjected to limited renaturation to generate types (a), (b), (c), and (d) molecules in each sample. The concentration of high- and low-abundance sequences is equalized among the type (a) molecules because reannealing is faster for the more abundant molecules. Consequently, type (a) molecules are significantly enriched for differentially expressed sequences. During the second hybridization, the two primary hybridization samples without denaturing are mixed together, and then the freshly denatured driver is synchronously added. The second hybridization should be carried out over a longer period to ensure that all complementary cDNAs became double stranded. When the reaction is completed, only the remaining equalized and subtracted ss tester cDNAs can form type (e) hybrids, which represent the differentially expressed genes between the tester and driver. Summarizing the entire population of molecules after two hybridizations, five kinds of products exist in the mixture, among which (e) molecules having two different adapter sequences at their 5′-ends that will allow exponential amplification in the following PCR cycles. No PCR reactions can be expected in the other two molecules of type (a) and (d) molecules because they have no primer-binding sites. Double-strand molecules of type (c), with only

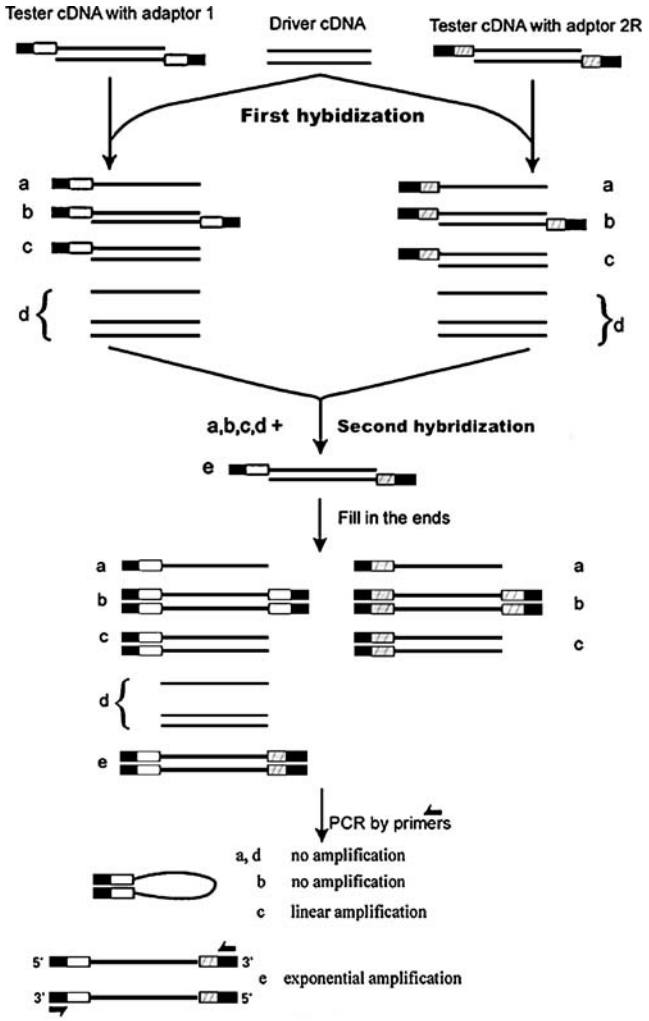

Fig. 1 Scheme of the SSH method. *Solid boxes*, *shaded boxes* and *clear boxes* represented the primers used in the first and second round PCR. In all products, **a** represented single strand DNA not hybridized in SSH; **b** represented molecules containing the same LITRs on both ends; **c** represented molecules having same expression between tester and driver; **d** represented molecules with no hybrid with tester; **e** represented the aim molecules with differential expression between tester and driver, and their resultant two different adapter sequences at 5′-ends allowing exponential amplification in PCR reactions

one adapter at one end, can only be amplified at a linear rate. Type (b) molecules contain the same LITRs on both ends and thus form stable "panhandle-like" structures after each denaturation–annealing PCR step. This resulting "panhandle-like" structure can prevent amplification in PCR reaction because intermolecular annealing of longer adapter sequences is both highly favored and more stable than intermolecular annealing of the much shorter PCR primers, which is the so-called suppression PCR effect. So after these two successive nested PCR, those upregulated genes in testers can be obtained and used for constructing a subtracted library. Working on the same theory, reverse SSH (switching the samples used as tester and driver) can find out the down-regulated genes in former testers.

Compared with other methods to detect differentially expressed genes such as the traditional cDNA subtractive hybridization, DD PCR, and RDA, SSH has its particular advantages. This method is more efficient for obtaining low abundance transcripts owing to the normalization in its procedure. For example, SSH to survey expression differences between Jurkat cells treated with phorbol 12-myristate 13-acetate (PMA) and untreated cells has identified six genes that previous systematic investigations missed using other methods (Gurskaya et al. 1996). Another detailed description on the efficacy of SSH was recently reported, in which two important conclusions were drawn under the assumption that hybridization followed the ideal second kinetic order (Wan et al. 2002). First, when target cDNAs are "all or nothing" of the differentially expressed genes, they can be enriched to a fixed level irrespective of its starting concentration. But to enrich quantitatively differentially expressed genes, it was deduced that the greater the expression ratio between the tester and driver, the more likely it is to obtain the target sequences by SSH. Aside from the advantage on efficacy, SSH requires smaller quantities of samples [1–2 μg poly(A)$^+$ RNA], and the process is relatively simple and fast. On the contrary, the traditional subtraction techniques often require greater than 20 μg of poly(A)$^+$ RNA, involve multiple or repeated subtraction steps, and are labor intensive.

However, the experimental procedure of SSH cannot exclude all nondifferentially expressed genes, meaning that some false positive clones representing nondifferentially expressed transcripts are often present in the library. In some cases, the number of false positive clones may considerably exceed the number of target clones in certain subtracted libraries. Rebrikov et al. (2000) suggested that the redundant cDNA molecules could contribute much to the occurrence of false positive clones, because they could evade elimination by hybridization with the driver and be amplified in subsequent PCRs. Meanwhile, the unincorporated adapters randomly annealed to nontarget cDNA molecules during subtractive hybridization that could raise the background to a certain extent. On the basis of this observation, they introduced some modifications such as the Mirror Orientation Selection technique to decrease the number of false positive (or background) clones in the libraries generated by SSH (Rebrikov et al. 2000). Their modification is based on the hypothesis that each species of background molecule has only one orientation relative to the adapter sequences. Thus, by removing one adapter, subsequent fresh heat denaturation and reannealing of the SSH samples, the newly formed hybrids bearing the residual adapter on both termini, will come from the target cDNAs with differentially expressed patterns and can be amplified exponentially. The other factor resulting in the deficiencies of SSH has been attributed to the absence of significantly differentially expressed genes

between the chosen driver and tester. That is, many false positive clones may be present in an SSH-subtracted library when in fact there was no dramatic alteration of gene expression between the two samples (Wan et al. 2002). On this theory, it was suggested that SSH favored highly differentially expressed genes but not genes important in the physiologically relevant processes with only minor changes. Despite these deficiencies, it is now obvious that SSH is a powerful approach to identify differentially expressed genes and can be widely applied to explore the molecular mechanisms involved in the complex process of physiology or pathology in living organisms.

Application of SSH in microbiological research

1. SSH for identifying differentially expression at mRNA level in eukaryotic microbes

It is known that diverse microbes play important roles in pathogenicity, fermentation for food or industry, and biogeochemical cycles. However, the genomes of some specific nonmodel microbes have not been fully completed, and it was reported that significant variation in gene content could be observed even between strains of the same species: 6–25% strain-specific genes had typically been found especially in pathogenic microbes (Fitzgerald and Musser 2001; Read et al. 2001). Having no prior requirement on sequence information, method SSH has become an important alternative of high throughput for studying the differences of gene expression in nonmodel microbiological research.

In eukaryotic microorganisms, especially in fungi, SSH has been employed to identify the differentially expressed genes at the mRNA level, similar to that for higher eukaryotes. Its application ranges from characteristic biologic behaviors such as sexual development (Wei et al. 2001), conidial formation and germination (Osherov et al. 2002), the differences of morphology with varying physical function (Dai et al. 2004; Dogra and Breuil 2004), to the molecular processes underlying disease development in important pathogens (Cramer and Lawrence 2004; Grenville-Briggs et al. 2005; Lu et al. 2005). To isolate genes with differential expression patterns between two samples, SSH is carried out firstly and the candidates are expected from the SSH-generated cDNA libraries. Then the analysis of Northern Blot or qPCR has to be performed to exclude the false positive clones. Finally, the validation of the roles played by the differentially expressed genes depends on the functional experiments, e.g. by gene knockout, antisense expression or overexpression.

An example for the application of SSH method in eukaryotic microbe research has been reported by Dai et al. (2004). It was demonstrated that the morphology of filamentous fungi including *Aspergillus niger* is crucial to the output of metabolic products in fermentation processes, and thus the ability to change or maintain a particular morphology is one of the key parameters in the development of productive fungal fermentations. However, the molecular mechanisms regulating the morphology of filamentous fungi remain largely unknown. To explore the genes involved in morphology determination, method SSH was employed before the genome of *A. niger* has been fully sequenced (Pel et al. 2007). This investigation aimed to identify the differentially expressed genes in *A. niger* associated with the switch from pelleted to filamentous growth, which accompanied a rapid decline in citric acid production. The screening of SSH-generated cDNA library and subsequent Northern blotting confirmed the different expression of 22 mRNAs from the estimated 14,000 total genes in *A. niger*. Fifteen genes were found highly expressed in the filamentous form, among which two were related to signal transduction pathways, six belonged to the group of amino acid synthesis and protein utilization, and there was no definite illustration to the functions of the remaining seven genes. Among the highly expressed genes in pallet cells, five of seven had no significant homology to the known sequences except that one was identical to the translated ubiquitin, and the other is homologous to an aspartic protease. To validate the potential roles of a filament-associated gene *Brsa-25* in controlling morphology and citric acid production (*Brsa-25*'s identifier number from *A. niger* genomic sequence is XP_001389968), a transformant with antisense expression was successfully constructed. The antisense strain showed an inhibition of filament formation and an increase in citrate production compared to its parent strain. These data from SSH suggested the possible mechanisms of morphogenesis and metabolite regulation, which might also be applied to construct the more powerful strains with genetic engineering.

2. SSH for identifying the different genomic sequences

Comparing genomic SSH with routine SSH used in eukaryotic cells at the mRNA level, some modifications in the protocols are necessary to yield higher efficiency according to the characters in genomic content. Akopyants et al. (1998) emphasized that three changes, while undesirable in eukaryote due to the existence of huge differences in mRNAs abundance, could improve performance and reliability in bacterial genome subtraction. First, DNA concentration can be reduced or polyethylene glycol (PEG), a hybridization enhancer used in cDNA subtraction for increasing the effective concentration of DNA in solution, can be omitted due to the low complexity of bacterial genomes. The absence of PEG also decreases the risk of fortuitous misannealing between different DNA molecules. Secondly, shortening the first hybridization time from 10 h to 1.5 h allows the increased amount of single-

stranded DNA that remains after the first subtractive hybridization and thereby makes the second hybridization more efficient. Lastly, a higher ratio of tester to driver DNA is needed in genomic SSH than that for eukaryotic mRNA. Furthermore, data from parallel experiments using different restriction enzymes in separate genomic SSH suggested a need to increase the coverage of positive clones significantly compared to using a single enzyme (Agron et al. 2001, 2002), while no available experimental evidences supported this viewpoint for the miscellaneousness in mRNA hybridization. When candidate DNA fragments are obtained, PCR amplification or Southern blot is required to confirm the results. Now it has been demonstrated that this modified SSH is effective for isolating the specific DNA region between different strains, biovars, serotypes, and species of bacteria based on the academic model and many experimental evidences.

One distinct purpose of using genomic SSH is to identify the different nucleotide sequences to serve as diagnostic markers for DNA-based detections (Radnedge et al. 2001; Agron et al. 2001, 2002; Dai et al. 2005). Some pathogenic bacteria can cause infectious disease outbreak, and thus rapid and effective identification of putative pathogens is necessary to eliminate nonpathogenic but closely related organisms. Such identifications would be more efficient if unique fragments in pathogen are available for designing specific PCR primers. For example, *Salmonella enterica* serovar Enteritides is a major cause of food-borne illness worldwide and a highly specific detection of this important pathogen requires the candidate DNA markers. Using *S. enterica* serovar Dublin as driver, some different regions were subtracted in *S. enterica* serovar Enteritides with genomic SSH, and these unique regions served as the pool for PCR primers. Among them, a pair of primers referred to as Sdf I (one region unique in S. enterica serovar Enteritides) was found effective in distinguishing serovar Enteritides from 73 non-Enteritidis isolates comprising 34 different serovars with real-time PCR (Agron et al. 2001). However, some DNA regions obtained by SSH have failed to be used as criterion for detection, although it is proved specific to the tester. DFR4, one region only present in *Yersinia pestis* biovar Antiqua, could be detected in only 57 of 60 Antiqua strains in natural plague foci, probably due to the sequence diversity or mutation (Dai et al. 2005).

The characterization of DNA content specific to individual strains that confers special phenotypes is illuminating for correlation between phenotypic differences and gene differences in prokaryotic microorganisms, such as in pathogenic vs nonpathogenic strains. Prokaryotic genome sequencing requires great effort and cost despite their relatively small genome. SSH, with no requirements on prior knowledge of genomic sequences, has become an increasingly important tool for comparative prokaryotic genomics. Pathogenicity islands (PAIs) are composed of the gene clusters closely related to pathogenesis and have drawn great interests from researchers endeavoring on the mechanisms and controls of infectious diseases. In recent years, successful cases have been carried out in some important pathogens with SSH method, including *Helicobacter pylori* (Akopyants et al. 1998), *Salmonella typhimurium* (Emmerth et al. 1999), *Escherichia coli* strain 536 (Janke et al. 2001) and *Burkholderia mallei* (DeShazer et al. 2001), in which novel PAIs with putative function of fimbrial biosynthesis, capsule export or translocation, DNA restrictive-modification enzymes and metabolic enzymes were isolated. These investigations will undoubtedly give some new and valuable implications on the pathogenesis or even the strategy of treatments.

Method SSH can also be applied to archaea, the most important inhabitants in the extreme environment. Using *Thermotoga maritima* MSB8 as a driver whose genomic sequences had been fully available, for example, SSH was performed in another close relative strain *Thermotoga* sp. RQ2 to detect genomic diversity (Nesbφ et al. 2002). Analysis within 426 sequenced clones from the constructed subtractive library revealed that about 40% (166) clones had no DNA match in the driver's genome, which corresponded to a total of 48 kb DNA and 72 RQ2-specific genes. Through a simple calculation, it was predicted that RQ2 might contain 350–400 specific genes on condition that strain MSB8 and RQ2 had the similar size genome. Experimental data also indicated that large numbers among these differential genes fell into three categories, including genes for producing surface structure, V-ATPase and a third novel archaeal type MutS homologs. Meanwhile, the other sequenced clones were shown to have a DNA match in *T. Maritima* MSB8: 130 clones shared the sequence identity of more than 85% and were classified as false positives; 93 clones were identified as divergent for their DNA identity of less than 85%. Then blot hybridization suggested that many differential or highly divergent genes in strain RQ2 have been acquired through lateral gene transfer from other members of *Thermotoga* sp. Thus, all these results proved SSH to be an easy and quick way to obtain differential genomic sequences in the closely related prokaryotic or archaea strains.

PCR-based positive hybridization

PCR-based positive hybridization (PPH) is a recently modified method based on the same principles of SSH with the opposite aim to identify the genes in common to different microbial strains. Thus the differences in the procedure of PPH are visible that no driver DNA is required and two tester DNAs ligated to a different terminal adaptor are hybridized at high stringency. When hybridization finishes, only those common

single-stranded sequences can reassociate to the new hybrids, and the latter can be amplified exponentially in the subsequent PCR reactions because they have both of the terminal adaptors annealing with PCR primers. Besides being used solely, PPH can also be integrated with SSH, that is to add a positive hybridization step after two successive hybridizations of SSH (subtracted PPH, Fig. 2). The extra step is to recover strain-specific genome fragments in common between different strains that have the same strain-specific property or activity.

It has been well described that the production of secondary metabolites is a valuable process for microbes, and those substances are generally of great importance for their special biological functions such as their potential use as insecticides and antibiotics. But the ability to synthesize bioactive metabolite is not universal to all strains in a species but limited to certain strains. Thus, PPH has been proposed as an alternative for studying the genetic basis of secondary metabolite production or the identification of genes that have been laterally transferred (Pomati and Neilan 2004). Because it is relatively new, PPH has been employed in only one study to isolate the common genomic DNA sequences associated with saxitoxin (STX) production between two toxigenic strains of cyanobacterium *Anabaena circinalis*. In this case, the efficacy and sensitiveness represented in PPH were assayed with the general aim for identifying genes potentially involved in STX biosynthesis and regulation. The results from subtracted PPH and microarry hybridization identified two toxin-specific clones common to toxic strains, while the other 11 clones were shown to be false positives or with no significant matches to the known cyanobacterial genomes. PPH of unsubtracted tester obtained seven toxic strain-specific clones in common to the tested toxin isolates. Analysis together with parallel data from SSH, unsubtracted PPH and subtracted PPH revealed that the subtracted PPH was less effective although the target fragments common to toxic strains at a low percentage could be obtained. The reasons contributing to the low efficacy and high false positives of subtracted PPH might be many as the final positive hybridization produced few clones for genes obtained from separate SSH experiments among the distinct strains. Subsequent PCR in PPH could not have sufficiently enriched sequences common to the two SSH libraries thus resulting in the high percentage of false positives in the library of subtracted PPH. So PPH using unsubtracted genomic libraries has been proven to be available in common sequences in different strains, supplying a possible method to acquire the information of cellular pathway related to the production of important metabolites.

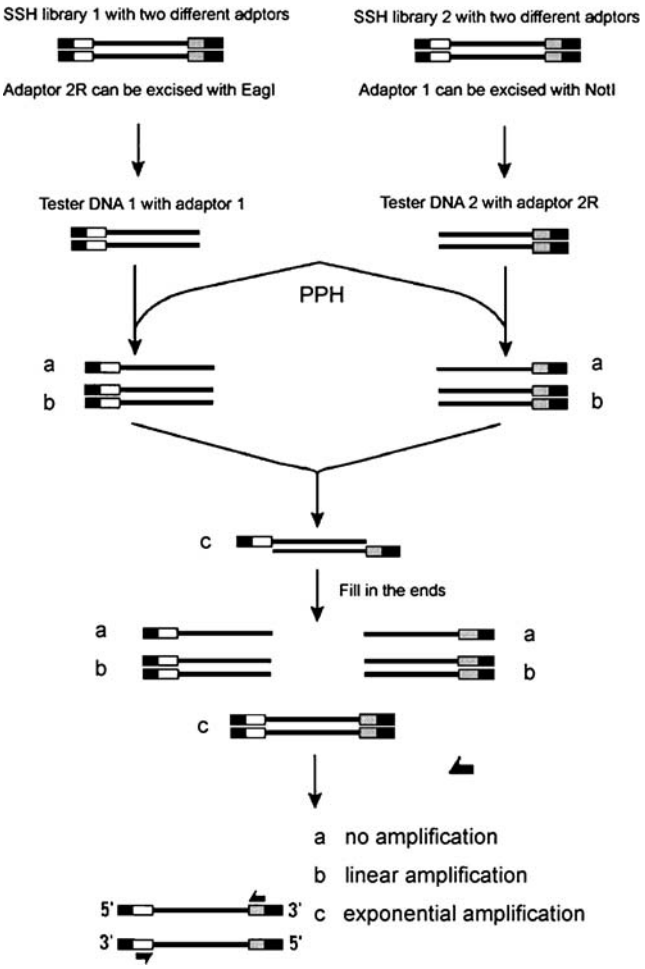

Fig. 2 The procedure of PPH was performed between two SSH libraries, in which adaptor 1 or 2R was removed in two tester DNA followed by the positive hybridization. Different boxes represent terminal adaptors 1 and 2R. **a** represented single strand DNA not hybridized in PPH; **b** represented molecules not having common expression between two tester DNA; **c** represented the aim molecules with common expression between tester 1 and tester 2, and their resultant two different adapter sequences at 5′-ends allowing exponential amplification in PCR reactions

Conclusion

The research on gene functions associated with biological activities or pathogenesis has become a focus after large quantities of sequencing work have been finished. SSH and its modifications, as a recently developed molecular genetic technique, can isolate either the genes differentially expressed at the mRNA level or genomic differences in among microbial strains, making it a useful tool in functional genomic studies.

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