

Development of novel vaccines using DNA shuffling and screening strategies

Christopher P Locher^{1*}, Nay Wei Soong¹, Robert G Whalen¹ & Juha Punnonen²

Address

Maxygen Inc

¹Division of Infectious Diseases

²Division of Immunotherapeutics

200 Penobscot Drive

Redwood City

CA 94063

USA

Email: christopher.locher@maxygen.com

*To whom correspondence should be addressed

Current Opinion in Molecular Therapeutics 2004 6(1):34-39

© Thomson Scientific ISSN 1464-8431

DNA shuffling and screening technologies recombine and evolve genes in vitro to rapidly obtain molecules with improved biological activity and fitness. In this way, genes from related strains are bred like plants or livestock and their successive progeny are selected. These technologies have also been called molecular breeding-directed molecular evolution. Recent developments in bioinformatics-assisted computer programs have facilitated the design, synthesis and analysis of DNA shuffled libraries of chimeric molecules. New applications in vaccine development are among the key features of DNA shuffling and screening technologies because genes from several strains or antigenic variants of pathogens can be recombined to create novel molecules capable of inducing immune responses that protect against infections by multiple strains of pathogens. In addition, molecules such as co-stimulatory molecules and cytokines have been evolved to have improved T-cell proliferation and cytokine production compared with the wild-type human molecules. These molecules can be used to immunomodulate vaccine responsiveness and have multiple applications in infectious diseases, cancer, allergy and autoimmunity. Moreover, DNA shuffling and screening technologies can facilitate process development of vaccine manufacturing through increased expression of recombinant polypeptides and viruses. Therefore, DNA shuffling and screening technologies can overcome some of the challenges that vaccine development currently faces.

Keywords Alphavirus, dengue, hepatitis, HIV, malaria, protein evolution

Introduction

DNA shuffling strategies encompass a variety of formats. In one format, DNA shuffling involves the recombination of genes *in vitro* to make novel proteins that are selected for improved functional properties. In this way, some of the fundamental steps of evolution are contained within this process, including mutagenesis, recombination, and selection for biological fitness. These technologies have been applied to solve problems in agriculture, chemical remediation and human therapeutics [1•]. Among the various DNA shuffling methodologies are multi-gene shuffling and synthetic shuffling. Shuffling of multi-genes (also known as family shuffling) typically involves recombining parental genes from a pool of related sequences [2], while DNA synthetic shuffling often involves creating multiple recombinations introduced from synthetic

oligonucleotides [3••]. Libraries of these chimeric polypeptides are then screened for improved functional properties or phenotypes, and have been utilized to address unmet needs in the protein pharmaceutical and vaccine industries [4]. In the case of vaccines, these characteristics or phenotypes can be improved cross-reactivity, immunogenicity and biological activity [5]. As novel chimeric clones with improved characteristics are selected in screening assays, prior knowledge of the protein structure is not necessary, although it can aid in the rational design of introducing diversity using synthetic DNA shuffling strategies [6]. For example, increasing the immunogenicity of a vaccine by rationally designing multiple amino acid substitutions within a specific domain may enhance vaccine efficacy. Therefore, DNA shuffling has the potential to solve complex problems in vaccine development using an evolutionary approach.

Recent developments in bioinformatics, statistical analyses, synthetic shuffling and robotic-mediated screening of libraries have made DNA shuffling and screening strategies more effective by improving library design for low homology shuffling, selecting specific domains to shuffle, introducing additional diversity and capturing a larger sample size of sequence space, respectively. This review demonstrates how DNA shuffling and screening strategies have improved vaccine candidates.

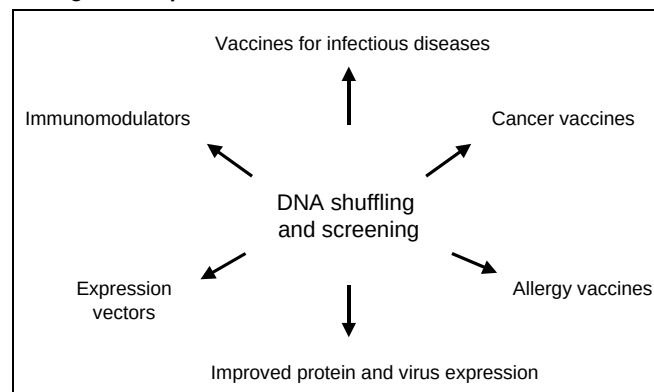
DNA shuffling

Natural evolution was accelerated using molecular biology when a relatively simple laboratory method was used to recombine DNA and select for improved progeny [7,8]. DNA shuffling and the associated screening technologies comprise a variety of formats [9]. In one format, chimeras generated by DNA shuffling are amplified using PCR technology. The process may begin with the selection of parental or starting genes from a group of related sequences. The starting genes are degraded by DNA fragmentation using specific enzymes that produce gene fragments. These gene fragments typically range from 5 to 10% of the original gene size and are then reassembled using DNA polymerase, which lengthens and extends the annealed gene fragments. During this assembly reaction, the genes are extended to their original size. These recombined genes contain portions or domains of the parental genes that are present in a multitude of combinations and are often highly chimeric.

The DNA shuffling reaction enables libraries of a very large size to be assembled (eg, 10^5 to 10^9 individual clones). Selected clones or hits from first round shuffling and screening are often re-shuffled among themselves to improve the biological fitness of selected chimeras [10,11]. Potential vaccine candidates can be screened for improved immunogenicity, cross-reactivity and/or stability, while retaining fundamental structure and function, such as receptor binding. Therefore, better vaccines can be developed using DNA shuffling and screening strategies

because progeny can be selected for their improved biological properties of cross-reactivity and/or immunogenicity. The broad applications of DNA shuffling and screening strategies are presented in Figure 1.

Figure 1. Applications of DNA shuffling and screening strategies to improve vaccines.



Vaccine applications have used DNA shuffling and screening strategies to generate chimeras that can be selected as vaccine candidates capable of inducing cross-reactive antibodies to multiple antigenic variants.

Bioinformatic strategies to assist library design, screening and analysis

Methods in the design of proteins, libraries and the optimizing of DNA shuffling reactions have substantially improved the success of identifying lead vaccine candidates. In addition to physical shuffling methods, bioinformatic approaches have been successfully applied to facilitate the identification of lead candidates. New mathematical algorithms explore sequence space of all of the possible combinations present in a family of libraries without a specific knowledge of the tertiary protein structure. Sequence space is all of the possible amino acids that can be generated by point mutations. For example, a small protein of 100 residues with 20 possible amino acids at a given position represents a sequence space of 20^{100} or 10^{130} combinations; however, only a small fraction of these proteins may yield a functional product that is biologically active. Protein sequence-activity relationship algorithm (ProSAR) is a statistical model that identifies residues predicted to be important with respect to biological activity [12]. ProSAR can identify the amino acid residues that contribute most to a desirable characteristic or higher fitness, for example, after DNA shuffling and screening. After analyzing these libraries, they can be designed to incorporate most of these amino acids for improving fitness, while at the same time minimizing amino acids that contribute to lowering fitness in subsequent rounds of DNA shuffling. As a result, shuffling of only key amino acids in a specifically described domain can increase the chances of identifying improved chimeras. Application of such bioinformatic approaches to library design can thus facilitate efficient searching of sequence space for desired molecules.

Various parameters and variables may influence the successful generation of chimeric sequences by DNA

shuffling, and these are usually determined empirically. These DNA shuffling parameters include salt concentrations, DNA polymerase, annealing temperature and conditions, in addition to the composition and complexity of DNA sequences. To optimize these conditions, computational analysis of the thermodynamics and kinetics of the DNA shuffling reaction has been shown to predict and improve the recombination process by improving the frequency of crossover events [13,14]. In these studies, the annealing thermodynamics considers the parameters of the reassembly step of annealing single-stranded DNA molecules and is useful for optimizing protocols in the DNA shuffling reactions. It is most useful for genes of < 2000 base pairs and can optimize the conditions for a successful shuffling reaction. An extension of these studies, SCRATCHY (a DNA shuffling method of libraries generated from ITCHY, incremental truncation for the creation of hybrid enzymes), has identified and optimized the conditions for increasing hybridization between heterologous genes having low homology and increased the number of crossovers [15]. Other methods for DNA shuffling techniques, including ITCHY, CLERY (combinatorial libraries enhanced by recombination), RACHITT (random chimerigenesis on transient templates), SHIPREC (sequence homology-independent protein recombination) and StEP (staggered extension process), as well as display screening strategies for libraries, have been reviewed [16•]. Each of these techniques has its own costs and benefits and multiple strategies can be used to evolve a specific protein in order to ensure success.

During the DNA shuffling process, recombination occurs in regions of greatest homology between parental sequences; therefore, blocks of parental sequences are usually conserved in the recombined genes from a shuffling reaction [13]. Synthetic shuffling strategies provide significant flexibility with respect to directing recombination. In a study by Ness *et al*, it was demonstrated that the amino acid diversity that was incorporated was far greater when genes of 15 subtilisin enzymes were synthesized with optimal codon usage and degenerate oligonucleotides were recombined in DNA shuffling reactions than in fragmentation-based DNA shuffling strategies [3••]. This study demonstrated that the greater flexibility of synthetic DNA shuffling reactions produces chimeras that would not have been generated by parental genes, as it expanded the accessibility of sequence space. Moreover, this study demonstrated that changes can be targeted to specific regions within a domain while retaining the native sequence of other domains of a polypeptide. Finally, another study demonstrated that recovery of parental sequences from distantly related β -xylanase genes is reduced in chimeric libraries when degenerate oligonucleotide gene shuffling (DOGS) is used for the gene assembly [17•]. It remains to be empirically determined if these synthetic DNA shuffling strategies will improve cross-reactivity and/or immunogenicity in appropriate animal models when applied in vaccine development.

New developments in library screening have also accelerated the identification of chimeras and determined overall library fitness. One study investigated the use of

multiprobe DNA microarrays to analyze the proportion of family-shuffled chimeric genes after *in vivo* recombination (CLERY; combinatorial libraries enhanced by recombination in yeast) [18]. In this study, fluorescent oligonucleotide probes were used for high-throughput sequence mapping and to assess library recombination biases. Similarly, probe hybridization was used in a gene chip format to screen libraries and to identify where specific crossovers took place; this demonstrated how biased the library was toward specific parental genes [19•]. When this method is applied using multiple probes for specific genes of low homology, the fitness and diversity of a library can be ascertained in the absence of directly sequencing multiple DNA clones.

Another method to rapidly screen libraries is to use an antibody tag to identify full length constructs and then sort bright-expressing clones using a fluorescence-activated cell sorter. As a supplemental approach, we used a dot blot assay to identify full-length clones expressing a C-terminal antibody tag [C Locher *et al*, unpublished data]. When used with a standard containing a known amount of protein, the dot blot method is used to identify clones that are expressed at high levels.

Improved receptor binding and higher affinity are important aspects in screening and selecting more effective vaccine candidates. Phage display has been useful to screen libraries of DNA-shuffled chimeras that have specific receptor affinity and can be useful in identifying specific clones that are potential malaria vaccine candidates. Ribosomal display is another technology that selects DNA-shuffled clones from libraries of chimeric proteins [20]. The efficiency of other screening methods based on display technologies has been reviewed (see reference [16•]).

Vaccine candidates for infectious diseases

An important application of DNA shuffling and screening strategies is to generate chimeric vaccine candidates that induce immune responses capable of controlling pathogens that have multiple antigenic variants or serotypes. There are numerous examples of such pathogens from viral, bacterial and parasitic infections that change their display of immunodominant antigens to adapt to the pressures of the host immune response (reviewed in references [21-23]). To overcome antigenic variation, some research groups have used a multivalent approach to incorporate antigens from several diverse strains and serotypes [24-26]. However, DNA shuffling strategies are more attractive because the

number of immunogens necessary to cover the antigenic diversity of a pathogen is reduced.

We have used DNA shuffling to generate chimeric vaccine candidates for multiple pathogens (Table 1). Shuffling of a family of genes was used to recombine the envelope genes of the four Dengue viruses and used to generate a chimeric vaccine candidate capable of inducing neutralizing antibodies to all four serotypes. Chimeras were selected using serotype-specific serum and then used to immunize mice. Serum from these demonstrated improved cross-reactivity to the four parental serotypes of Dengue, as measured in enzyme linked immunosorbant assay (ELISA) and virus neutralization assays.

A synthetic DNA shuffling and screening strategy was also used to generate chimeras from an important malaria vaccine candidate that undergoes antigenic variation, *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP-1). This protein is expressed on the surface of the malaria parasite as part of the knob protrusions, and mediates pathogenesis by binding to multiple receptors [27]. Moreover, antibodies to the surface of infected red blood cells are associated with resistance to disease [28] and PfEMP-1 protects against experimental malaria in non-human primates when the cysteine-rich interdomain region (CIDR)-1 α is used as a vaccine antigen [29]. After DNA shuffling of the CIDR-1 α , cross-reactive antibodies measured by ELISA were induced more effectively in mice immunized with the shuffled chimeric proteins than in mice immunized with the parental constructs. These data further conclude that DNA shuffling and screening methods represent important approaches to facilitate immunity against pathogens that undergo antigenic variation.

In addition to generating cross-protective vaccines, genes from specific vaccine antigens can be shuffled to improve immunogenicity. This was successfully demonstrated with the improvement of a shuffled hepatitis B virus (HBV) surface antigen in both DNA and protein vaccine formats [5]. DNA shuffling strategies may be used to evolve antigens with improved affinity to antibodies, in addition to antigens that are more efficiently processed and presented in the context of class I and class II major histocompatibility complex (MHC) molecules. Screening strategies for improved immunogenicity in transgenic mouse models should be helpful to identify lead candidates with improved capacity to induce cytotoxic T-lymphocytes (CTLs) and T-cell recall responses.

Table 1. DNA shuffling and screening strategies to evolve vaccine candidates.

Target organism	Evolved antigen	Goal of DNA shuffling and screening
Viruses		
Dengue virus	Envelope	Cross-protection against four serotypes
Alphaviruses	Envelope (E1 and E2)	Cross-protection against three viruses
HBV	Surface antigen	Improved immunogenicity
HIV-1	Envelope (gp120)	Improved neutralizing antibodies
Bacterium		
<i>Staphylococcus</i>	Enterotoxin	Cross-neutralization
Parasitic protozoan		
Malaria	PfEMP-1	Cross-protection against antigenic variants
<i>Plasmodium falciparum</i>		

Alphaviruses include Venezuelan, western and eastern equine encephalitis viruses. **HBV** Hepatitis B virus, **HIV-1** human immunodeficiency virus, **PfEMP-1** *Plasmodium falciparum* erythrocyte membrane protein-1.

DNA shuffling and screening also allow for the selection of improved antigens for therapeutic vaccines against cancer and allergy. One of the major limitations of cancer vaccines is the fact that the host immune system is typically tolerant to the tumor-associated antigens that are used as candidate vaccines. DNA shuffling and appropriate screening methods allow for the selection of antigens that are dissimilar enough to the self-antigen that they escape the immunological tolerance mechanisms, yet are similar enough to induce a strong cross-reactive humoral and cellular immune response against the tumor antigen. On the other hand, in the case of allergy vaccines, anaphylactic allergic reactions triggered by the vaccine itself have limited vaccine use and dosing. DNA shuffling and screening methods enabled the generation of chimeric recombinant allergens with reduced binding to patient IgE, thereby resulting in allergy vaccine candidates with potentially improved safety profiles.

Improving immune responses with genetic adjuvants

Co-stimulatory molecules expressed on antigen-presenting cells play a key role in inducing and modulating T-cell activation, proliferation and effector functions. Among the most important and widely studied co-stimulatory molecules are B7-1 (CD80) and B7-2 (CD86), which enhance or inhibit T-cell function via interaction with CD28 or CTLA-4, respectively. An increase in immune responses (antibody titers and CTL activity) has been demonstrated when B7-1 and B7-2 molecules are administered with vaccine candidates [30-32]. To determine if DNA shuffling and screening strategies could evolve new co-stimulatory molecules with improved properties, B7-1 molecules sharing genetic homology were recombined from multiple mammalian species [33••]. A shuffled co-stimulatory molecule, termed CD28 binding protein (BP), strongly improved T-cell proliferation and interferon (IFN) γ production upon co-cultivation with human T-cells; CD28BP also demonstrated improved binding to CD28 when compared with wild-type human B7-1. In contrast, transfectants expressing another shuffled co-stimulatory molecule, CTLA-4BP, increased interleukin (IL)-10 secretion and inhibited T-cell proliferation in mixed lymphocyte reactions in a dose-dependent manner. Co-administration of shuffled CD28BP with vaccine candidates may improve T-helper type 1 (Th1) function, including recall and CTL function. On the other hand, CTLA-4BP has potential in applications of allergy and autoimmunity where diminished T-cell responses are desirable.

IL-12 is a cytokine that has an adjuvant effect in multiple infectious diseases, facilitates tumor abolishment in animal models, and is an essential regulator of Th1 responses [34]. To evolve a cytokine with improved adjuvant effects, homologous genes from seven mammalian species encoding the p35 and p40 subunits of IL-12 were shuffled [35••]. One clone, evolved (Ev)IL-12, demonstrated 10-fold improved expression in mammalian cells, in addition to a 10-fold increase in specific activity in inducing human T-cell proliferation when compared with wild-type human IL-12. Based on the sequence analysis of EvIL-12, the amino acid changes in the chimeric cytokine are likely to facilitate

heterodimer formation, which may be one of the reasons that, when compared with human IL-12, improved expression levels were observed. EvIL-12 may be more effective as a vaccine adjuvant and a therapeutic molecule than human IL-12, due to its increased biological activity and a potential lower therapeutic dose.

Improvement of vaccine manufacturing by directed evolution

DNA shuffling and screening strategies can be applied to generate viruses with improved replication kinetics, viral envelope stability and altered tropism. Viral adaptation methodologies may achieve these aims, but because DNA shuffling can bring together disparate viral strains from unique evolutionary pathways, it is likely to make improvements more quickly and efficiently than natural evolution. DNA shuffling and screening strategies have evolved an HIV-1 variant that can replicate in pig-tailed macaque peripheral blood mononuclear cells. These new variants of HIV-1 are likely to benefit the development of a macaque model for the evaluation of HIV-1 vaccines, as the use of chimpanzees, the only non-human primate that can be infected with HIV-1, is severely limited [36••]. This study demonstrated that viral tropism can be changed by DNA shuffling and that viral variants can be selected by specific screening assays. Nevertheless, as DNA shuffling may be used to change viral tropism, judicious experimental design and careful risk assessments must be performed and reviewed with an institutional biosafety committee using the National Institute of Health's Recombinant Advisory Committee guidelines. In addition, altered cell tropism and improved envelope viral stability were demonstrated in a separate study following DNA shuffling of murine leukemia virus used in gene therapy applications [37]. This increased stability in the functional integrity of the retroviral envelope is useful in enhancing the yields, titers and purity of vector preparations during the manufacturing process. These successful demonstrations of the ability of DNA shuffling and screening strategies to tailor viral phenotypes suggest that such strategies may be applied to evolving other useful phenotypes into vaccine virus strains. Examples are stability to purification processes and temperature, and compatibility with formulations that can enhance their production and shelf-life. Selecting for adaptation to advantageous cell lines, changes in growth conditions (such as reduced reliance on bovine serum), or for improved yields in the current production cell line represent other potential routes where manufacturing efficiencies can be realized. The careful design of selection criteria can facilitate the identification of desired characteristics while eliminating the non-desired characteristics. Other strategies such as whole genome shuffling by protoplast fusion may also be useful in vaccine development by combining various bacterial serotypes and selecting their ability to replicate [38•].

When recombinant polypeptide vaccines are expressed, aggregates in host cells sometimes form due to protein insolubility, making the protein purification process difficult. To address this problem, DNA shuffling and screening methods may be applied to increase the amount of recombinant polypeptide expression for vaccine production. For example, DNA shuffling and screening approaches can

be applied to promoter sequences for improvements in the regulation of protein expression, inducing greater stability of mRNA structure or incorporating hydrophilic and acidic amino acid residues. In summary, DNA shuffling and screening strategies have the potential to be applied to solving expression and manufacturing problems of numerous vaccine candidates.

Conclusion

New bioinformatic developments have accelerated the development of molecular breeding-directed molecular evolution by providing tools to facilitate the design, synthesis and analysis of DNA-shuffled libraries. These developments, coupled with display technologies and high-throughput screening, have provided effective methods to generate and identify chimeric proteins that are conformationally correct and have improved affinity or altered specificity to their receptors. By combining evolved genetic adjuvants and immunomodulatory molecules with DNA-shuffled cross-reactive antigens, new candidate vaccines with improved immunogenicity and stability have been generated. DNA shuffling and screening technologies are likely to improve both preventative and therapeutic vaccines for cancer, allergy, autoimmunity and infectious diseases. Moreover, it is believed that these technologies will enable the generation of completely new vaccines for diseases and disorders for which no vaccines or immunotherapies are currently available.

Acknowledgements

We are grateful to all the scientists at Maxygen whose contributions have enriched the development of DNA shuffling and screening methodologies. Particular thanks for their input to this review go to Peggy Powers, Sharon Fujita and Aaron Kurtzman. This research was supported partly by grants from Defense Advanced Research Projects Agency and the United States Agency for International Development.

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