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Signature-tagged mutagenesis: technical advances in a negative selection method for virulence gene identification

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Signature-tagged mutagenesis (STM) is a powerful negative selection method, predominantly used to identify the genes of a pathogen that are required for the successful colonization of an animal host. Since its first description a decade ago, STM has been applied to screen a vast amount of transposon insertion mutants in 31 bacterial species. This has led to the identification of over 1700 bacterial genes that are involved in virulence. Despite the preservation of the basic design, the STM method has been developed further owing to recent advances including different designs of the signature-tags and profound changes in the mode of detection. These advances promoted substantially the application range and versatility of the STM method.

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Introduction

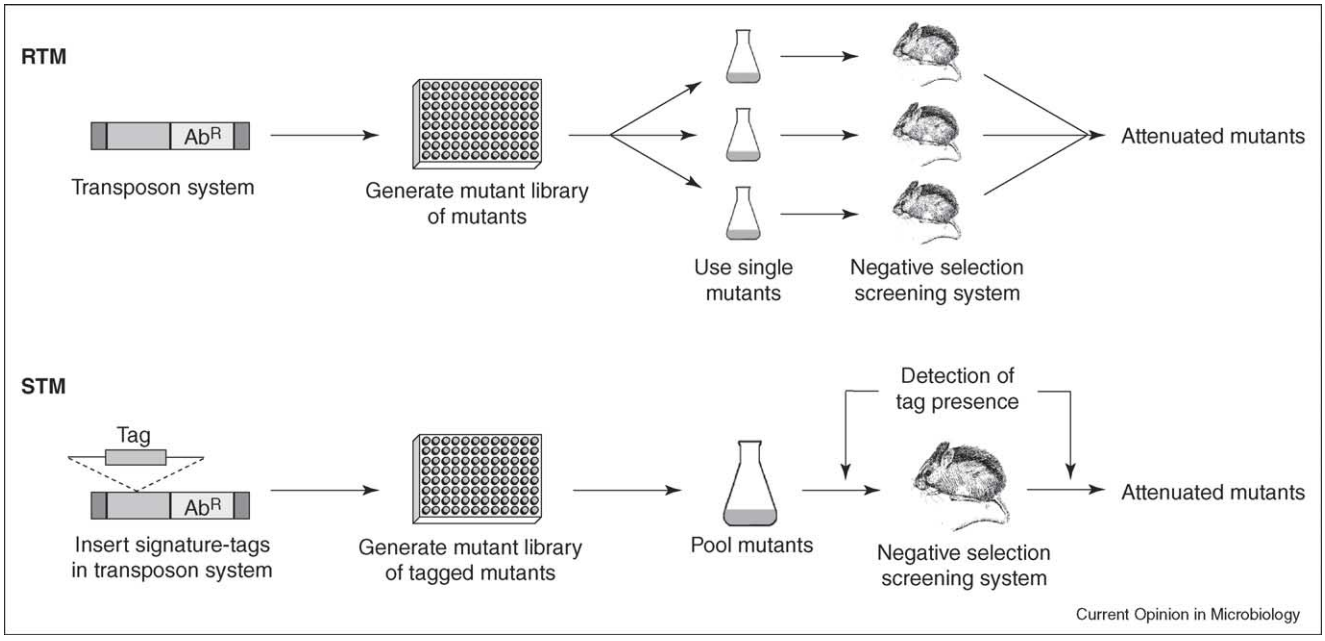
The availability of complete genome sequences for most bacterial pathogens increased substantially the number of genes with unknown function. Genome-wide approaches to functionally characterize these genes in the process of infection have become of great importance. Gene-disruption strategies, such as random transposon mutagenesis, produce insertion mutants that can be tested for attenuated virulence (e.g. in an animal infection model). The isolation of attenuated mutants thus leads to the identification of genes or operons that are required for survival in the infected host. Before the invention of signature-tagged mutagenesis (STM) ten years ago by David Holden and co-workers [1], these mutants had to be screened one by one; however, STM combines the power of insertional mutagenesis and negative selection with a detection system, which allows one to identify individual attenuated mutants from a complex mutant pool (Figure 1). To this end, STM uses signature-tags (i.e. short individual DNA sequences) inserted in the transposons to mark mutants individually. Mutants that carry distinct signature-tags are pooled and injected into the animal host to test in parallel for their survival. This is advantageous as it minimizes both the work-load and the number of animals required.

Owing to its frequent application, STM has been reviewed extensively in recent years [2–7]. Review articles have compared technical variations in STM studies [5], have presented limitations of the STM approach [2,3], and have summarized the results of STM studies until 2000 [4] and 2001 [6]. In this review, we highlight the modular structure of this powerful negative selection method and focus on the technical advances since 2002.

Modular structure of the signature-tagged mutagenesis approach

For the purpose of this review, we present STM as a flowchart of interconnected modules, as depicted in Figure 2. The original STM approach described by Hensel *et al.* [1] was designed to detect new virulence genes of the target organism *Salmonella typhimurium* in a murine model of typhoid fever. To this end, miniTn5 transposons that contained signature-tags composed of random sequences of 40 bp were randomly inserted into the bacterial genome, yielding a tagged mutant library. To validate the suitability of individual tags for detection within a pool of differently tagged mutants, single mutants were pooled and used for test hybridizations. Therefore, the tags of a pool were polymerase chain reaction (PCR)-amplified with universal tag primers, radioactively labeled, and hybridized on membranes spotted with DNA from the corresponding mutants. Only mutants with clear tag hybridization signals were included in the subsequent selection process. Input pools of 96 mutants were subjected to a negative selection system, in this case a mouse infection model. The corresponding output pools recovered after selection were grown on complex medium and their tags amplified and labeled for detection. A weak or absent hybridization signal from the output pool compared to the input pool identified attenuated mutants (Figure 3a). These mutants were tested by different means (e.g. for competition with wild-type bacteria in mixed infections) to validate the screening results. Identification of the mutation site by cloning and sequencing revealed known virulence genes, but also genes previously unrelated to virulence and those with unknown function. Most strikingly, further characterization of selected mutants led to the discovery of a novel *Salmonella* pathogenicity island (SPI-2) [8].

Figure 1



Comparison of standard random transposon mutagenesis (RTM) and signature-tagged mutagenesis (STM), displaying similarities and differences between these two methods.

Since this initial STM study, numerous STM screens have followed a similar protocol. Modifications within individual modules have increased the versatility of the STM method. Some target organisms, such as *Neisseria meningitidis*, are refractory to transposon mutagenesis, leading Sun *et al.* [9] to use *in vitro* mutagenesis and homologous recombination to assemble the tagged mutant library. Other STM studies have used two different negative selection systems [10] or have re-screened to validate their initial screen results by constructing new pools with attenuated mutants and submitting them to a second screen under the same or similar conditions as in the initial screen [11]. In addition, profound changes have been made to some of the modules of the STM screen. For tag validation, Mei *et al.* [12] introduced pre-selection of tags that showed reproducible detection and no cross-reactivity. Each tagged transposon could be subsequently used separately to generate a large amount of tagged mutants. Many STM studies adopted this procedure or directly used the pre-selected tags from previous studies, facilitating the establishment of the method for the specific needs of the study. Also, the way in which mutants are detected has changed profoundly from the original STM methodology. Lehoux *et al.* [13] introduced PCR detection instead of hybridization (Figure 3b).

Recent advances in signature-tagged mutagenesis

In recent years, many new STM studies have been carried out (Table 1). We summarize the major technical changes in the different modules of these STM studies (Table 2).

Target organism

Although most STM studies examine pathogen–host interactions, the method is not limited to this application. One recent study investigated the symbiont–host interaction for *Xenorhabdus nematophila* in its nematode host *Steinernema carpocapsae* [14] and another studied the commensal–host interaction for *Campylobacter jejuni* in chicken [15•].

Transposon

Most studies to date have applied the miniTn*5* transposon system [16], which was used in the original STM screen [1] for tag-delivery and mutation of the chosen target organism. This system works in γ-Proteobacteria, among others, but as host factors are required for transposition and owing to target DNA composition, some bacteria are (nearly) refractory to random mutagenesis by Tn*5*-derived transposons. For this reason, several recent studies in *Streptococcus pneumoniae*, *N. meningitidis* and *C. jejuni* [15•,17,18] applied transposons from the *mariner* family, such as *magellan2* or *Himar1* [19]. The activity of these transposons is not dependent on host factors and thus they are applicable to a broad variety of organisms, and only the respective transposase is needed for *in vitro* transposition [20]. The high frequency of transposition and the low insertion-site specificity render these transposons ideal for random transposon mutagenesis [21].