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The prince and the pauper: Which one is real?

The problem of secondary mutation during mutagenesis in *Streptococcus pyogenes*

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It has been known in the community studying the pathogenesis of *Streptococcus pyogenes* that spontaneous mutation occurs during passages in laboratory media or in animals, so some of the progeny's virulence can be changed, mainly due to the change of the expression of virulence factors.[1-3] This phenomenon is not only limited to *S. pyogenes*, but also happens to other pathogens.[4-6] Like other pathogens, *S. pyogenes*, a strict human pathogen, produces virulence factors to attach to and invade host tissues, avoid the immune system, or degrade or kill host tissues and cells. These virulence factors are necessary to colonize, invade, and spread in the host. However, some of these factors are not necessary to grow in laboratory media, so reduced expression of these virulence factors could increase the fitness for growth in laboratory media. A notorious *S. pyogenes* virulence factor whose expression changes during passages in the laboratory is M protein. M protein is a cell wall-anchored protein with a coiled-coil structure and its important role in *S. pyogenes* pathogenesis has long been recognized as an adhesin and antiphagocytic factor.[7] It is also the causative molecule of the streptococcal rheumatic heart disease. The antibodies generated against M protein cross react with tissues in the heart valves.[8] Thus, when *S. pyogenes* loses M protein, its virulence is dramatically attenuated in animal infection models.

It has been a puzzle in the study of *S. pyogenes* whether or not the *sagA/pel* locus influences the expression of the M protein gene (*emm*). Several studies on the *sagA/pel* locus produced conflicting results on the regulation of M protein gene expression. However, the recent study by Zhou et al. in this issue of *Virulence* shows that the *sagA/pel* locus does not regulate the expression of M protein at least in the M1 strains that most studies for the *sagA/pel* locus used, and the confusion has been caused by secondary spurious mutations during mutagenesis.[9] This study by Zhou et al. reminds us of the importance of awareness of spontaneous mutation or phase variation possibilities during mutagenesis of *S. pyogenes*. During laboratory passages, the expression of other virulence factors such as capsule and the protease SpeB changes,[3] so this phenomenon seems not to be limited to M protein.

Recent genomic studies show that genetic changes in *S. pyogenes* occur not only during passages in the laboratory (in vitro passage) but also during infection (in vivo passage).[10-13] Interestingly, these genetic changes are selected mostly in the virulence factor-regulator genes of *mga*, *covRS*, and *ropB*. In the study by Zhou et al., the expression of both genes of M protein and its regulator Mga decreased in strains producing low levels of M protein after lab passages, and there was no mutation in the *emm* and *mga* promoter region. However, since the expression of *mga* is autoregulated,[14] it cannot be ruled out that Mga was mutated in the study. The major virulence factors regulated by Mga, CovRS, and RopB are M protein, capsule, and SpeB, which are the same virulence factors whose expression was changed after laboratory passages. Therefore, the selected mutations during in vitro passages might also be concentrated in these regulators.

To prevent improper interpretation of a function attributed to a gene due to spurious secondary mutations, mutagenesis should be carefully performed and the phenotype of mutants should be carefully examined. *S. pyogenes* starts to die quickly after reaching the stationary phase in THY medium (most popular lab medium for *S. pyogenes*) probably due to the lack of nutrients, low pH (~pH 5.5) etc. It has been known that cells go through more mutation during conditions not favorable for growth such as in the stationary phase.[15,16] Thus, during mutagenesis, cells should not stay in the stationary phase too long. After mutagenesis, the phenotype of several mutant colonies, not just one or two, should be examined. The expression of the virulence factors M protein, capsule, and SpeB can be easily examined phenotypically. The production level of capsule is easily determined by the appearance and size of colonies on agar plates. The expression level of the major secreted protease SpeB can be examined by plating cells on skim milk agar plates.[17] Most, if not all, *S. pyogenes* strains form aggregates during stationary phase in liquid media, and M protein is involved in the aggregate formation through

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homophilic protein–protein interactions.[18] However, other cell surface proteins such as M-like proteins, Protein H, and Protein F are also responsible for the aggregation. Thus, it is wise not to pick non-aggregated colonies if other colonies are aggregated in liquid media. However, when the expression of M protein needs to be tested, a direct method such as western blotting should be performed.

Another way to confirm the function of a gene attributed after mutagenesis is to perform the complementation test. Most commonly, multicopy plasmids are used to complement a gene or genes in *S. pyogenes*. Complementation with a multicopy plasmid is easy to perform, and if it works, the gene can be manipulated easily by deleting a motif or changing key amino acids through inverse PCR. However, complementation with a multicopy plasmid does not work in some cases, perhaps due to the issues of the level or the location of expression. Another way to perform complementation is to insert the same gene using a suicide vector into the mutagenized site. If an intact gene is introduced into the original site in the chromosome, then the problems of copy number and expression location can be solved. Using this method, we succeeded in complementing genes whose complementation with a multicopy plasmid did not work.[17,19] Previously, McShan et al. developed a streptococcus suicide vector system that is capable of site-specific integration.[20] This vector system contains *S. pyogenes* phage genes to facilitate the vector's integration into a serine tRNA gene, so this system can also be a useful system for complementation with a single copy gene.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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