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Pitfalls encountered while investigating genetic elements by PCR

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The unprecedented wealth of databases that have become available in the era of next-generation sequencing has considerably increased our knowledge of bacterial genetic elements (GEs). At the same time, the advent of single-cell based approaches has brought realization that unsuspected heterogeneity may occur in the bacterial population from a single colony. The increasing use of PCR-based techniques to study new GEs requires careful consideration of the possible different PCR targets associated with different subpopulations if incorrect or incomplete interpretations are to be avoided. In this commentary, confining ourselves to our direct experience, we illustrate some examples of PCR pitfalls that may be encountered while investigating GEs.

Keywords: genetic elements, PCR mapping, PCR pitfalls, single-cell based approaches, Intra-colony heterogeneity

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In recent years the advent of next-generation sequencing technology has considerably advanced our knowledge of genetic elements (GEs). At the same time, the development and refinement of single-cell based approaches[1] has been showing that the traditional notion of bacterial cells growing in liquid cultures or on plates as myriads of largely identical cells is an outmoded oversimplification:[2] indeed, even a single bacterial colony may display significant heterogeneity, since a majority of genetically identical cells can grow side by side with variably sized subpopulations exhibiting substantial genotypic (and occasionally phenotypic) differences. Among the many implications of these advances, we would like to highlight how some molecular methods commonly used in the study of bacterial GEs—specifically PCR-based techniques—need careful monitoring and verification to avoid incorrect or incomplete interpretations.

It is not a new piece of knowledge, of course, that an integrative GE (such as a phage or an ICE) may be excised in circular form from the host chromosome, for instance as the first step of transfer. However, only in recent years has it finally come to be realized that this entails the coexistence of three PCR targets in the bacterial genome: (1) the chromosome with the integrated GE; (2) the excised GE in circular form; and (3) the chromosome alone, repaired after GE excision. Albeit found in significantly different amounts, each can trigger the amplification reaction without distinction.

This enhanced awareness has in some instances enabled earlier results to be reconsidered and reinterpreted. Confining ourselves to the experience with streptococcal GEs made by the researchers of our group, a case in point has been our route to the characterization of ϕm46.1, the widespread *Streptococcus pyogenes* phage carrying *mef*(A) and *tet*(O) resistance genes.[3] Since its first demonstration,[4] the PCR-based linkage between the two genes was conceived of from a linear perspective, with *tet*(O) located ~5.5 kb upstream of *mef*(A).[5-7] Subsequently, however, it was realized that the ~5.5 kb between the genes had been measured from the circular form, whereas in the more common integrated form *tet*(O) is found close to the right end and *mef*(A) is found upstream, close to the left end of the prophage: in fact > 46 kb apart (too distant for an amplification reaction).[3] Now, thanks to this new insight, an old problem has become a new resource, since comparing PCR results obtained from the

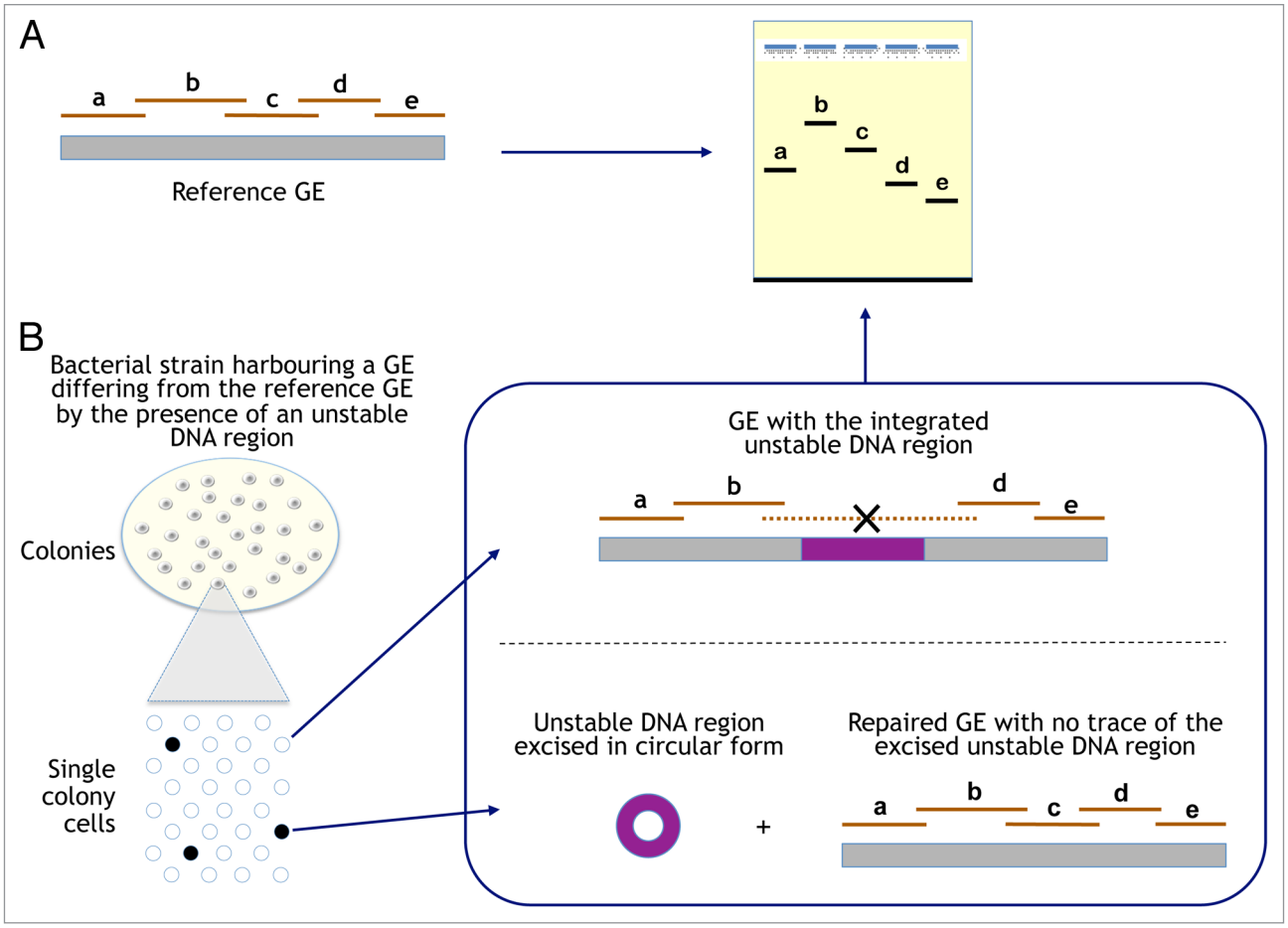

Figure 1. PCR mapping of (**A**) the reference GE and (**B**) the GE being investigated, which differs from the former by the presence of an unstable DNA region. The results obtained from the GE being investigated will overlap with those from the reference GE, i.e., they will fail to highlight the unstable DNA region, integrated in the GE, carried by the majority of bacterial cells. Indeed, the failure of PCR mapping to yield band c in this majority of cells (because the distance is excessive for amplification or because of the PCR mapping strategy adopted) will be concealed by the band c yielded by the minority of cells where the unstable region has been excised in circular form.

three targets adds an unhoped-for working tool in GE investigation, for instance in the determination of the chromosomal integration site of a new GE.

The unprecedented wealth of today's databases has considerably simplified GE search and characterization, and PCR mapping—possibly integrated with restriction analysis and/or sequencing—is among the most extensively used investigational approaches. However, an additional source of complexity results from recent studies indicating that, in the bacterial population, GEs may display further heterogeneity due to unstable DNA regions capable of undergoing excision in circular form. Such regions may be other GEs—e.g., an ICE integrated into another[8]—or even unconventional circularizable structures.[9] Anyway, the bacterial population will encompass two distinct cell subpopulations, one bearing the unstable region integrated in the GE and the other lacking the unstable region, whose excision has left no trace in the repaired GE. The latter subpopulation, which is likely to make up a minority of cells in the colony, may provide a positive result of the PCR mapping assay, thus concealing the lack of amplification in the majority of cells (**Fig. 1**). Still confining ourselves to our direct experience, this is the case of 15K, described in *Streptococcus suis* ICE*Ssu32457*.[10] This unconventional circularizable structure could be detected just because it contained drug resistance determinants that were the starting point of the investigation: had the study started from the 'container' rather than the 'content', PCR mapping would almost certainly have hidden the unstable DNA region (too large to yield a PCR reaction). In the case of smaller unstable structures, such as the MAS element from *Streptococcus pneumoniae* Tn*1545*/Tn*6003*,[11-13] the detection—though possible in theory—will depend in practice on the mapping strategy and conditions applied.

The question is thus what information PCR mapping does actually supply. Based on the traditional concept that a GE is uniform in the bacterial population, a positive PCR mapping reaction was regarded as a milestone, i.e., as confirming that the element being investigated and the reference element shared an identical genetic organization; conversely, a negative reaction or a PCR product of unexpected size prompted the search for a possible cause of diversity (e.g., an insertion or a deletion). Now,

however, the knowledge about unstable DNA regions adds a new, critical factor in that, failing quantitative information (achievable, for instance, by appropriate Southern blotting assays), a positive PCR mapping reaction might be deceptive and not preventable even by amplicon restriction analysis and/or sequencing. In some instances PCR mapping will therefore provide inconclusive GE characterization, since an underestimation of GE size or cargo content cannot be excluded.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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