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Molecular Aspects

Mycobacterium bovis BCG moreau is naturally deficient in homologous recombination

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ABSTRACT

The ability to perform genetic manipulation of mycobacteria is important for characterization of gene function. Homologous recombination-based protocols are frequently used for reverse genetics studies with mycobacteria. It is known that *Mycobacterium bovis* BCG Russia, closely related to *M. bovis* BCG Moreau, is a natural *recA* deficient strain and is non-permissive to homologous recombination assays. In this work we show that *M. bovis* BCG Moreau is also deficient in homologous recombination, shown by a specialized transduction assay, but this phenotype can be reverted by complementation with heterologous recombinases, using a recombineering protocol. Sequence analysis of the genes known to be involved in homologous recombination annotated in the genome of BCG Moreau detected no differences compared to the genome of BCG Pasteur. Further studies are needed in order to determine the exact mechanism underlying this deficiency in BCG Moreau.

1. Introduction

Tuberculosis is the first infectious contagious disease in death numbers worldwide and its causative agent is *Mycobacterium tuberculosis* (*M.tb.*) [1]. Among the mycobacteria there are other pathogenic species, such as *M. leprae*, also a human pathogen, and *M. bovis*, a cattle and human opportunistic pathogen, as well as a number of free-living organisms. All of them share common biochemical and genetic features, such as a lipid-focused metabolism and a highly complex cell wall, as well as high illegitimate recombination (IR) rates [2]. Several groups focus on unraveling mycobacterial biology, aiming to develop better prophylactic and/or therapeutic strategies to deal with diseases caused by these bacteria. For that, laboratory cultivation and manipulation of cell machinery are necessary. In this aspect, *M. leprae* represents an example of a laboratory non-cultivable mycobacteria, dependent on a host for cell replication.

As a highly complex and distinct group, several molecular biology and genetic studies were developed to understand its metabolic and regulatory pathways, often focusing on inactivation of specific genes and complementation [3]. Using this approach one can infer biological function, comparing the phenotypes of wild-type, knockout and complemented strains in respect to a specific characteristic. The main strategies to delete specific genes are homologous recombination (HR)-based, where, in the majority of cases, an allelic exchange substrate (AES) is created harboring a selectable marker flanked by regions of homology to the targeted gene. Initial studies were based on the use of suicide plasmids bearing the cloned AES and transformation of the desired mycobacteria followed by selection for the presence of the marker. In this strategy, HR events depend on the cell's own recombinases. More recent protocols rely on specialized transduction [4], increasing AES transformation efficiency, or recombineering [5], increasing recombination rate by providing highly efficient recombinases from mycobacteriophages.

In Brazil many studies focus on *M. bovis* BCG Moreau [6,7], the strain used for vaccine production, either as a *M.tb.* model or for gaining knowledge on vaccinal features. But efforts to generate knockout mutants in this strain through traditional homologous recombination were unsuccessful (Odir Dellagostin, personal communication). Here we report the results of a study using BCG strains Moreau and Pasteur, comparing the capacity of generating knockouts for two different

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operons. Our results show that BCG Moreau has a deficiency in homologous recombination, and that this phenotype can be circumvented by supplementing cells with phage recombinases functionally similar to λ Exo and Beta proteins. This indicates that this deficiency is specific to the initial steps of the whole process and not due to a mutation in *recA* itself, since the BCG Moreau sequence is identical to both the *M.tb.* and BCG Pasteur functional genes.

2. Material and methods

2.1. Bacterial cultivation

M. bovis BCG Moreau and Pasteur were cultivated on 7H9 liquid or 7H11 solid media supplemented with 10% albumin/dextrose/catalase (ADC) or oleic acid/albumin/dextrose/catalase (OADC) mixture, respectively. *Escherichia coli* strains and *M. smegmatis* mc^2155 were grown on Luria Bertani (LB) medium, with addition of 0.05% (v/v) Tween 80 to the latter. We used 37 °C and 30 °C as incubation temperatures to non-permissive (knockout creation assay) and permissive conditions (plaque lysis detection), respectively.

2.2. Allelic exchange substrate construction

We first created the allelic exchange substrate (AES) to construct *rv1371/2/3* and *rv1552/3/4/5* homologues knockouts on *M. bovis* BCG Moreau and Pasteur. To do so, we amplified fragments containing sequences of ~1 kb upstream and downstream to the desired locus to be excised using BCG Moreau genomic DNA, and then used them to flank a kanamycin resistance gene (*kanR*) by performing a fusion PCR. These final sequences were amplified and used in recombineering assays. These AES were also sub-cloned on a PvuI-digested pUC18, disrupting the plasmid's ampicillin resistance gene (*ampR*). This was done to enable the transfer of the AES to phAE87 mycobacteriophage *ampR* by HR on heat-induced *E. coli* DY380 cells. After transforming phage-harboring DY380 cells with recombinant plasmids, recombination events were selected by plating on kanamycin (40 μg/mL) at 30 °C and recombinant phage structure was confirmed by PacI digestion. Selected phage DNAs were then transformed on *M. smegmatis* mc^2155 and after incubation on permissive conditions phage particles were collected, titrated and this suspension was used on specialized transduction assays.

2.3. Specialized transduction and recombineering

For specialized transduction, phage suspensions with 10^8–10^9 phages/mL were used to infect BCG Moreau and Pasteur. We incubated phages with bacterial cells for 3 h prior to plating. For recombineering, around 150 ng of purified AES was used to transform competent acetamide-induced pJV53H-harboring BCG Moreau and Pasteur cells. Before selection on kanamycin plates, transformed cells were incubated for 24 h on medium without antibiotics on respective temperatures. Each allelic exchange protocol was performed three times for each condition.

2.4. Verification of locus genetic structures by PCR

Locus excision was confirmed by amplifying intralocus sequences (wild-type genotype) and by using primers that flank locus-peripheric sequences and *kanR* (knockout genotype). For the wild-type genotype analysis we used the following pairs of primers for *rv1371/2/3* (forward: ATGAACGTCTCAGCTGAGAG and reverse: AATTGACCACGACACGTGATA) and *rv1552/3/4/5* homologues (frd9: AAAGACATGGAATTCGTCCAAT and frd10: CGTTGATATCGGTGTGAACG). For the knockout genotype we used KmF (GCCATCCTATGGAACTGCC) and KmR (GCCTAGAGCAAGACGTTTCC) as *kanR* annealing primers. With KmF we used TCCGCAGACCCTAATACAC and TGTGGCATCCACGTCCAGAT (frd6) as reverse primers, and with KmR we used TTGGATAACAAAGGCTGAACAT and GGTGGACCTTGCATCAGATG (frd1) as forward primers, for *rv1371/2/3* and *rv1552/3/4/5* homologues knockout structure verification, respectively. Amplification products were analyzed by 1.0% agarose gel electrophoresis and visualized after ethidium bromide staining (Fig. 1).

3. Results and discussion

M. bovis BCG Moreau has been the strain used in Brazil for TB vaccine production for over 90 years and has been studied by many Brazilian scientific groups. However, major advances on understanding its molecular operating mechanisms have been hampered by its probable HR deficiency. In this work we looked deeper into this issue, with a specific outlined experimental set, aiming to confirm this information and to detail the steps responsible for BCG Moreau's deficit.

We used BCG Pasteur, one of the most studied BCG strains, as a positive control for recombination events, since it has been used successfully for gene knockout experiments [8]. Two distinct and separate operons were chosen as test sequences to be deleted: the *frd* operon, coding for the fumarate reductase complex, involved in anaerobic respiration, and *rv1371/2/3* orthologs, possibly encoding a lipid-producing system, since Rv1372 and Rv1373 are described as an alpha-pyrone polyketide synthase, Pks18, and a glycolipid sulfotransferase, respectively. The choice of two different unrelated loci aimed at minimizing false negative results due to specific structures of the chromosome region. In order to analyze if this HR-deficient phenotype is related to the lack of specific recombinases, the recombination assays were performed with addition of heterologous phage recombinases, via recombineering [5] or without, via specialized transduction [4]. These techniques were chosen since they were available and standardized in the laboratory and are less time consuming than plasmid-based methods.

After selection by plating with specific antibiotic we detected similar numbers of resistant colonies for both bacterial strains, using both techniques and with the two AESs. Locus structure was checked by PCR, as schematically shown in Fig. 1. True knockouts yielded DNA fragments of 1175 and 1177 bp, with primers annealing on the kanamycin resistance gene and on sequences 1 Kb up/downstream (UP/DOWN) relative to the sequence to be excised, respectively. False knockouts, on the other hand, only amplified with primers annealing on the wild-type allele yielding a 424 bp DNA fragment. Ten isolated colonies from each experimental condition were checked by PCR confirming the true knockout genotype for BCG Pasteur in all conditions and colonies tested. For BCG Moreau, only colonies resulting from the use of the recombineering protocol showed the true knockout genotype, while all colonies resulting from the use of the specialized transduction protocol had a false-knockout genotype. The phage ability to infect BCG Moreau was analyzed by lysis plaque detection on permissive infection and culture conditions, with similar results between Pasteur and Moreau strains, showing that differences on allelic exchange rate obtained with both strains using the specialized transduction protocol were not due to strain-related differences on transduction efficiency. These results show that allelic exchange is only accomplished on BCG Moreau when heterologous recombinases are supplied to the system, as is the case with the use of the recombineering protocol. It is important to point out that in the specialized transduction protocol, phage (phAE87) particles harboring the AES on its DNA is used simply as a vector, enhancing the process's transformation efficiency in comparison to a traditional electroporation protocol, the most used mycobacterial transformation technique. Therefore, the resistant BCG Moreau colonies resulting from the use of this phage-delivery system represent IR events. High rates of IR is a known and described characteristic of slow-growing mycobacteria [2], so it is not surprising that, in the absence of a proper HR machinery and under selective pressure, resistant colonies arise derived from this process.

This HR-deficient phenotype has been described for BCG Russia [9],

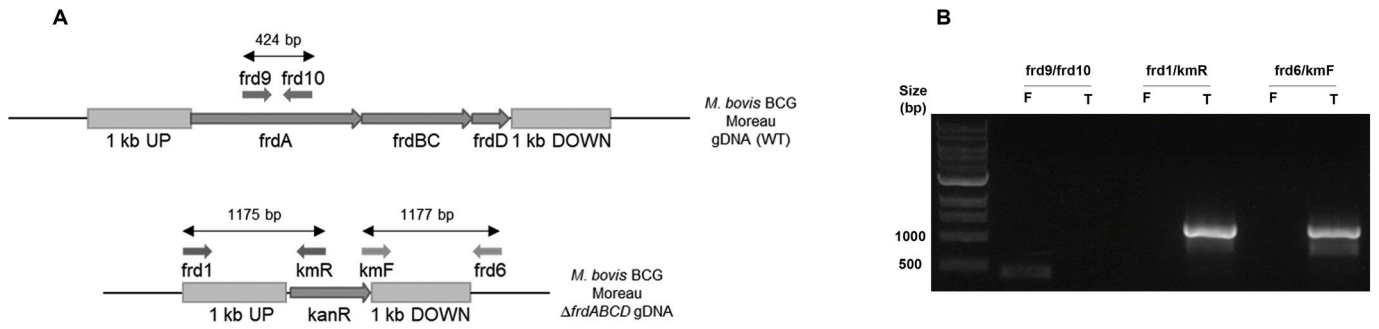

Fig. 1. Summary of assays used to asses BCG Moreau homologous recombination machinery efficiency. Two distinct loci were used (*rv1371/2/3* and *frdABCD*). (A) Locus structure, represented by *frdABCD*, of both wild-type (WT) and Δ*frdABCD* strains, indicating the pair of primers used for genetic structure assessment. Ten resulting colonies from each condition were chosen to verify the locus structure by PCR and products analyzed by agarose gel electrophoresis (representative gel shown in panel B) to detect true (T) and false (F) knockouts.

a phylogenetically close strain to BCG Moreau, that together with BCG Tokyo or Japan are part of BCG's DU2 Group I [10]. This characteristic is often associated with a high degree of genome conservation over time, since HR and other recombination processes that share enzymes with the former would periodically aid genome restructuration [11]. Thus, this phenotype is a desired feature for a biopharmaceutical, as it would not significantly alter its own characteristics and prophylactic effects over time. Regarding BCG Russia, it is known that this event is *recA*-related, as this strain carries a one-base insertion on this gene leading to a frameshift mutation and the appearance of a premature stop codon, yielding a much shorter mutated protein (139 amino acids long instead of 790, as the *M.tb* homologue). This leads to a polypeptide lacking the native C-terminal part responsible for DNA binding, probably hampering its HR-related activity. However, under DNA-damaging conditions, BCG Russia also failed to express RecA indicating that this mutation might not be the only factor responsible for its HR deficient phenotype [9].

Analysis of BCG Moreau *recA* using its available genome sequence (GenBank accession number: **AM412059**) [6] shows it to be identical to the gene in BCG Pasteur and *M.tb*. Therefore, this feature alone does not explain our results, indicating that the *recA* mutation in BCG Russia results from a specific event (synapomorphy) not shared by other members of the DU2 Group I clade. To further probe the possible effectors involved in this HR-deficient phenotype we looked at other genes known to be involved in the process of HR or its regulation (*adnA*, *adnB*, *recF*, *recO*, *recR*, *ruvA*, *ruvB*, *ruvC*, *recJ*, *recN* and *recX*) [12], comparing the sequences from *M. bovis*, *M. bovis* BCG Moreau, BCG Pasteur and *M. tb*. No differences were found, except for *adnA* and *recF*. In the former, the product differs from Rv3202c on A748V and in the latter, from Rv0003 on V158L and T245I. In both cases, the mutation was a common event among BCG Moreau and Pasteur and does not explain BCG Moreau's HR-deficient phenotype since Pasteur has normal HR rates. Even though no significant differences in the primary sequence of these genes were identified, a supplemental expression study is needed in order to further clarify if this homologous recombination deficient phenotype could result from factors related to transcription regulation.

We also analyzed *recB*, *recC* and *recD*, even though in mycobacteria, unlike *E. coli*, RecBCD is involved on the pre-synaptic step of another repair mechanism called single-strand annealing (SSA) [13]. In the *M. bovis* lineage *recB* is frameshifted due to a single base deletion yielding a shorter product with a different C-terminal part compared to the *M.tb.* ortholog. Furthermore, these genes seem to be organized in an operon in the C–B-D order with possible translational coupling between *recB-D* due to overlapping stop-start codons [14], which would be disrupted by *recB*'s premature stop. With that we can infer that the *M. bovis* lineage is deficient in RecBCD activity, an end-resection nuclease involved in SSA DNA repair pathway. Further work is necessary to assess the biological relevance of this finding.

As already described [12] HR can be divided in three major steps: pre-synaptic (*adnA*, *adnB*, *recF*, *recO*, *recR*, *recJ*) comprising break end resection and RecA loading on the derived single strand; recombination (*recA*); and resolution of Holliday junctions (*ruvA*, *ruvB*, *ruvC*). Our recombineering assays employed pJV53, a plasmid that yields acetamide-inducible Gp60 and Gp61 expression, two mycobacteriophage (Che9c)-derived recombinases functionally similar to λ Exo and Beta proteins. These enzymes participate in initial recombination steps such as annealing of complementary DNA strands, strand exchange and invasion [5]. The final resolution step is dependent on the bacterial own enzymatic machinery. Contrary to the *E. coli* Red system in which other λ phage genes are present [15], in the mycobacterial recombineering system using pJV53 only the recombinases involved in the first two steps of HR are present, leaving solely for bacterial resolvases to deal with the last step. So, our results clearly show that the BCG Moreau lack of HR is related either to the initial pre-synaptic step, or to a deficient expression or activity of RecA, in spite of the absence of a *recA* deleterious mutation.

3.1. Conclusions

It is important to emphasize that our work confirms a long-time known fact, namely that BCG Moreau is not amenable to homologous recombination, but still does not clarify which genes are responsible for this deficiency. Our *in silico* results show no evidence of mutation on HR-related genes, but further analysis should be performed to detect transcriptional regulatory effects that may hamper the overall process. Complementation of the natural HR knockouts represented by BCG strains Moreau and Russia could be used to understand if these HR-deficient phenotypes are evolutionary linked.

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References

[1] World Health Organization. Global tuberculosis report 2018. 2018.
[2] Muttucumaru N, Parish T. The molecular biology of recombination in mycobacteria: what do we know and how can we use it? Curr Issues Mol Biol 2004; 6:145–58. https://doi.org/10.21775/cimb.006.145.
[3] van Kessel JC, Marinelli LJ, Hatfull GF. Recombineering mycobacteria and their phages. Nat Rev Microbiol 2008;6:851–7. https://doi.org/10.1038/nrmicro2014.
[4] Bardarov S, Bardarov S, Pavelka MS, Sambandamurthy V, Larsen M, Tufariello J, et al. Specialized transduction: an efficient method for generating marked and unmarked targeted gene disruptions in Mycobacterium tuberculosis, M. bovis BCG

and M. smegmatis. Microbiology 2002;148:3007–17. https://doi.org/10.1099/00221287-148-10-3007.
[5] van Kessel JC, Hatfull GF. Recombineering in Mycobacterium tuberculosis. Nat Methods 2007;4:147–52. https://doi.org/10.1038/nmeth996.
[6] Gomes LHF, Otto TD, Vasconcellos EA, Ferrao PM, Maia RM, Moreira AS, et al. Genome sequence of Mycobacterium bovis BCG Moreau, the Brazilian vaccine strain against tuberculosis. J Bacteriol 2011;193:5600–1. https://doi.org/10.1128/JB.05827-11.
[7] Pagani TD, Guimarães ACR, Waghabi MC, Corrêa PR, Kalume DE, Berrêdo-Pinho M, et al. Exploring the potential role of moonlighting function of the surface-associated proteins from Mycobacterium bovis BCG Moreau and Pasteur by comparative proteomic. Front Immunol 2019;10. https://doi.org/10.3389/fimmu.2019.00716.
[8] Ramón-García S, Mick V, Dainese E, Martín C, Thompson CJ, de Rossi E, et al. Functional and genetic characterization of the tap efflux pump in Mycobacterium bovis BCG. Antimicrob Agents Chemother 2012;56:2074–83. https://doi.org/10.1128/AAC.05946-11.
[9] Keller PM, Böttger EC, Sander P. Tuberculosis vaccine strain Mycobacterium bovis BCG Russia is a natural recA mutant. BMC Microbiol 2008;8:120. https://doi.org/10.1186/1471-2180-8-120.
[10] Brosch R, Gordon Sv, Garnier T, Eiglmeier K, Frigui W, Valenti P, et al. Genome plasticity of BCG and impact on vaccine efficacy. Proc Natl Acad Sci Unit States Am 2007;104:5596–601. https://doi.org/10.1073/pnas.0700869104.
[11] Dixit PD, Pang TY, Maslov S. Recombination-driven genome evolution and stability of bacterial species. Genetics 2017;207:281–95. https://doi.org/10.1534/genetics.117.300061.
[12] Singh A, Bhagavat R, Vijayan M, Chandra N. A comparative analysis of the DNA recombination repair pathway in mycobacterial genomes. Tuberculosis 2016;99:109–19. https://doi.org/10.1016/j.tube.2016.04.011.
[13] Gupta R, Barkan D, Redelman-Sidi G, Shuman S, Glickman MS. Mycobacteria exploit three genetically distinct DNA double-strand break repair pathways. Mol Microbiol 2011;79:316–30. https://doi.org/10.1111/j.1365-2958.2010.07463.x.
[14] Huber M, Faure G, Laass S, Kolbe E, Seitz K, Wehrheim C, et al. Translational coupling via termination-reinitiation in archaea and bacteria. Nat Commun 2019;10:4006. https://doi.org/10.1038/s41467-019-11999-9.
[15] Poteete AR. What makes the bacteriophage Ĩ» Red system useful for genetic engineering: molecular mechanism and biological function. FEMS (Fed Eur Microbiol Soc) Microbiol Lett 2001;201:9–14. https://doi.org/10.1111/j.1574-6968.2001.tb10725.x.