

Short communication

Instability of the acetamide-inducible expression vector pJAM2 in *Mycobacterium tuberculosis*

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Abstract

The *Escherichia coli*-mycobacterium shuttle vector pJAM2 has been used to inducibly express genes in mycobacteria. The vector carries the promoter region from the highly inducible acetamidase gene of *Mycobacterium smegmatis* which is used to drive expression of heterologous genes. We used pJAM2 to over-express the *Mycobacterium tuberculosis* gene Rv2868c, a homologue of *gcpE*. In *M. smegmatis* the plasmid was stable, but the promoter region was readily deleted when the parental vector or recombinant plasmids were transformed into *M. tuberculosis*. We mapped the deletion by sequencing and found that it encompassed the entire acetamidase promoter and adjacent sequence totalling approximately 7.3 kb and occurred very soon after introduction into *M. tuberculosis*. This is the first report of instability of a vector carrying the acetamidase promoter in *M. tuberculosis*.

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The World Health Organisation (WHO) has estimated that eight million individuals annually are newly infected with *Mycobacterium tuberculosis*, the main causative agent of human tuberculosis, resulting in some two million deaths per year (Dye et al., 1999). Currently the only available vaccine is the live, attenuated bacillus Calmette–Guerin

(BCG) strain; however, it does not offer complete immunity and protection is highly variable due to a wide range of social, economic, and environmental factors. Current research trends are focused on the production of an improved vaccine, identification of new drug targets and the development of new anti-mycobacterials. All of these activities have benefited greatly from the availability of the complete genome sequence of *M. tuberculosis* (Cole et al., 1998).

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The use of expression vectors to express proteins as antigenic compounds is essential for the production of new vaccines or for functional studies. The *Escherichia coli*-mycobacterium shuttle vector pJAM2 was developed for the high level inducible expression of genes in mycobacteria (Triccas et al., 1998). Expression is under the control of a ~1.5 kb fragment derived from the upstream region of the *Mycobacterium smegmatis* acetamidase gene (Mahenthiralingam et al., 1993; Parish et al., 1997). This region contains three regulatory genes and several promoters (Parish et al., 1997, 2001; Roberts et al., 2003), although the precise mechanism of induction has not been fully elucidated. The system is induced by the addition of acetamide to the growth media (Draper, 1967) and has been used in a number of settings including over-expression of genes, antisense expression, and conditional expression of essential genes (Greendyke et al., 2002; Manabe et al., 1999; Payton et al., 1999). The advantage of using inducible vectors for over-expression is that it allows for direct comparisons between the induced and non-induced conditions in strains carrying a single plasmid. It is also assumed that inducible vectors are more stable than constitutively expressing vectors, such as those utilising *hsp60* or *sodA* gene promoters (DeSmet et al., 1999). Sequencing of *M. tuberculosis* has revealed that it does not contain the acetamidase gene or promoter region (Cole et al., 1998). pJAM2 has orf regions 1, 2, and 5 from origin of replication of the *Mycobacterium fortuitum* plasmid pAL5000 (Stolt and Stoker, 1996) allowing it to replicate in *M. tuberculosis*. As *M. tuberculosis* is an ACDP category three pathogen and is also particularly slow growing, with a typical doubling time of 24 h, a non-pathogenic faster growing (doubling time typically 2 h) related species, *M. smegmatis*, is often employed as a model organism. We were interested in using pJAM2 to over-express a number of key genes involved in metabolic pathways. Our first gene of interest was the *M. tuberculosis* homologue of *gcpE* (Rv2868c, GenBank Accession No. CAA15530), which encodes an enzyme involved in the early steps of isoprenoid biosynthesis. We attempted to over express the *M. tuberculosis gcpE* in both *M. tuberculosis* and *M. smegmatis*.

We constructed a *gcpE* overexpression vector by amplifying the *M. tuberculosis gcpE* gene as a 1.3 kb fragment using primers gcpE-pJ-F (5'-GG ATCTAGAGTGACTGTAGGCTTGGG-3') and gcpE-pJ-Rev (5'-GACTCTAGAGAACTCCCTT TGGTGGGAACT-3') and cloned it into pJAM2 to make pJAMA1. We electroporated pJAMA1 and pJAM2 into both *M. smegmatis* and *M. tuberculosis* as previously described (Parish et al., 1999). *M. tuberculosis* (H37Rv) was grown on Middlebrook 7H10 agar (18 g/L Middlebrook 7H10, supplemented with 10 % v/v Middlebrook oleic acid–albumin–dextrose–catalase enrichment (OADC)) [Becton–Dickinson]; *M. smegmatis* (mc²155) was grown on Lemco medium (Roberts et al., 2003). Transformants were selected on agar containing 20 µg/ml kanamycin.

The *M. tuberculosis* transformants had an unusual phenotype. Initially transformants of pJAMA1, and pJAM2 were very slow growing, taking 6 weeks to form colonies, rather than the usual 3–4 weeks. Transformants were inoculated into 10 ml liquid medium (4.7 g/L Middlebrook 7H9 broth, 10% v/v OADC, and 0.05% w/v Tween 80) and grown standing at 37 °C. One milliliter from these cultures was used to inoculate 10 ml of fresh media every 4 weeks. After the second sub-culturing, the *M. tuberculosis* transformants showed a noticeably improved growth rate and grew to higher ODs in a shorter period of time. Unusually this phenotype was seen with the strains transformed with both the parental and recombinant plasmids, so it could not be due simply to stress from over-expression of *gcpE*. In contrast the *M. smegmatis* transformants appeared normally after 3 days and grew normally in liquid media.

Plasmid instability has been reported in mycobacteria previously, particularly in *M. smegmatis* and *M. bovis* BCG (Al-Zarouni and Dale, 2002; Chawla and DasGupta, 1999; DeSmet et al., 1999; Kumar et al., 1998). The carriage of plasmids poses an extra pressure on cells and it is possible that rearrangements or deletions could be occurring in the cells; if such changes partially relieved the selective pressure then we would expect them to accumulate in the population over time. This could account for the initial slow growth of the plasmid-

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