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Abstract: *Burkholderia pseudomallei* is the causative agent of melioidosis, a potentially fatal disease endemic or emerging world-wide. Here we report efficient unmarked allele-replacement mutagenesis using efficient *sacB* counter-selection. Despite being genotypically *sacB*⁺, most commonly used *B. pseudomallei* strains are sucrose-resistant and efficient *sacB* counter-selection is demonstrated in both resistant and sensitive strains.

Dear editor

Please find attached a Short Communication (Note) for publication in J. Microbiological Methods.

We believe the observations and conditions described in the paper to be extremely useful to the research community undertaking 'knockout' mutagenesis with *B. pseudomallei* and addresses many concerns about this method.

Yours sincerely,

Ifor Beacham

1 Facile construction of unmarked deletion mutants in *Burkholderia pseudomallei* using *sacB*
2 counter-selection in sucrose-resistant and sucrose-sensitive isolates.
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1
2 **Abstract**
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7 *Burkholderia pseudomallei* is the causative agent of melioidosis, a potentially fatal disease
8 endemic or emerging world-wide. Here we report efficient unmarked allele-replacement
9 mutagenesis using efficient *sacB* counter-selection. Despite being genotypically *sacB*⁺, most
10 commonly used *B. pseudomallei* strains are sucrose-resistant and efficient *sacB* counter-
11 selection is demonstrated in both resistant and sensitive strains.
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24 Keywords: *sacB*; counter-selection; unmarked deletions; *Burkholderia pseudomallei*
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2 *Burkholderia pseudomallei* is a Gram-negative, aerobic, saprophytic, bacillus classified as a
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4 potential agent for bioterrorism (category B select agent; (CDC 2008)). It causes a
5
6 broad-spectrum of diseases in humans and animals, collectively termed melioidosis.
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8
9 Melioidosis is associated with a high mortality rate and a high rate of relapse and can affect
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11 almost every host organ (Currie et al., 2000). The septicaemic form of melioidosis usually
12
13 involves a rapid onset and death within 24-48 hours and has the highest mortality rate
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15 (Wiersinga et al., 2006). Infection occurs through inhalation, ingestion or percutaneous
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17 acquisition of bacteria and is prevalent in patients with underlying conditions such as diabetes
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19 and renal disease. The endemic area of melioidosis is limited to tropical and sub-tropical areas
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21 with the major foci being Southeast Asia and northern Australia (Leelarasamee and
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23 Bovornkitti 1989; Dance 1991) and melioidosis has been suggested to be an emerging disease
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25 worldwide (CDC 2008).
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31 The annotated sequence of the genome of *B. pseudomallei* K96243 (Holden et al., 2004),
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33 and more recently the sequences of other isolates, have recently become available, allowing
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35 investigations of the genetic basis of virulence in this enigmatic pathogen. Although a few
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37 genes associated with virulence have been identified (Wiersinga et al., 2006), it seems likely
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39 that the virulence of *B. pseudomallei* is also combinatorial involving a number of virulence-
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41 associated genes and thus multiple deletions may need to be made in one strain in order to
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43 assess virulence. Furthermore, the genome of *B. pseudomallei* is characterised by the presence
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45 of multiple genomic islands which vary amongst strains (Brown and Beacham, 2000; Holden
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47 et al., 2004; Sim et al., 2008). The deletion of these islands will be helpful in establishing their
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49 possible role in virulence. This requires an efficient method of mutagenesis and such deletions
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51 are preferably not marked by selectable cassettes which might themselves compromise
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1 virulence (Knodler et al. 2005) or reduce the ability to mutate more than a single gene in one
2 isolate.
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4 One pathway for allele replacement mutagenesis is integration of a suicide vector
5 containing a deleted allele of the locus by homologous recombination, followed by excision
6 of the integrated vector in the merodiploid in a second recombinational event. This latter step
7 requires counter-selection for the vector. *sacB*-mediated counter-selection is based on the
8 presence of *sacBR* in a suicide vector, rendering an integrant phenotypically sucrose-sensitive
9 (Ried and Collmer, 1987). However, the genome of *Burkholderia pseudomallei* isolates for
10 which genome sequence is available notably contains a *sacB* gene (BPSS0543; Holden et al.,
11 2004). This predicts that *B. pseudomallei* would already be sucrose-sensitive, obviating the
12 use of *sacB* vectors in allele replacement. Although *sacB* –mediated mutagenesis has been
13 reported for *B. pseudomallei*, it is problematical and consequently many studies have used
14 potentially polar single cross-over mutants.
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31 Alternative counter-selection methods have been devised, and used in mutagenesis to
32 counter-select vectors either in the second (vector excision) recombinational step in *B.*
33 *thailandensis* or to counter-select *flp*-encoding vectors in *B. pseudomallei* (Choi et al., 2008;
34 Barrett et al., 2008). The latter is required for excision of *FRT* cassettes which may be used in
35 selecting for initial merodiploid formation. The use of selectable cassettes leaves a
36 recombination site (“scar”) which is potentially polar, although the mutated allele can be
37 constructed such that the reading frame is maintained (Choi et al., 2008). Transformation
38 using linear PCR fragments has also been reported, but requires the use of selectable cassettes
39 which, again, can be excised using *FLP-FRT* recombination; notably, transformation with
40 PCR fragments is unsuccessful with some *B. pseudomallei* strains such as K96243 (Thongdee
41 et al., 2008).
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1 Here we assess the utility of the widely used and simple *sacB* counter-selection method for
2 the generation of unmarked deletion mutants in *B. pseudomallei*. We report that most
3 commonly used *B. pseudomallei* strains are not in fact sensitive to sucrose, despite encoding a
4 *sacB* gene, and hence efficient *sacB* counter-selection is demonstrated under optimised
5 conditions. Such conditions are described for both sucrose-resistant and sucrose-sensitive
6 isolates. The method allows multiple deletions mutants, both of single genes and larger
7 multigenic loci, to be efficiently and simply generated in any strain, in turn facilitating
8 investigation of the genetic basis of *B. pseudomallei* virulence.
9

10 The first step in mutagenesis requires the construction of a deleted allele of the gene, or
11 target multigenic region, to be deleted. Upstream and downstream DNA fragments of
12 approximately 1 kb are amplified with appropriate primer-pairs, ligated together and re-
13 amplified with the upstream forward and downstream reverse primers. The resulting 2 kb
14 fragment is cloned into the suicide vector pDM4 (Milton et al., 1996) or pDM4-Tp, a
15 derivative of pDM4 with a trimethoprim-resistance cassette cloned into the *SalI* site
16 (unpublished data). This construct is then transferred to *B. pseudomallei* by conjugation with
17 selection for the merodiploid as chloramphenicol and/or trimethoprim resistance; the
18 advantage of selection for two markers is that background antibiotic-resistant clones are
19 eliminated by dual selection or by testing for simultaneous trimethoprim resistance. This
20 ensures that the clones are due to integration and not mutation to antibiotic resistance (which
21 occurs at significant frequency at least in the case of chloramphenicol resistance; data not
22 shown) and do not need to be distinguished by PCR analysis. Selection for the second cross-
23 over is by selection for sucrose-resistance followed by screening for chloramphenicol and/or
24 trimethoprim-sensitivity, indicating that the suicide vector, pDM4 or pDM4-Tp, had been
25 excised from the genome in the second recombinational event (see Ried and Collmer, 1987).
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1 We tested a number of *B. pseudomallei* isolates for sucrose-sensitivity under conditions
2 which were optimised for sucrose-sensitivity in merodiploids (see below). Saturated cultures
3 (1-3x10⁹/ml) were plated at 10⁻¹, 10⁻², 10⁻⁴, and 10⁻⁶ dilutions. After 48 hrs incubation, these
4 isolates fall into two clear groups: those either naturally phenotypically sucrose-sensitive,
5 even at the highest cell density, and those sucrose-resistant (Table 1), with the majority being
6 sucrose-resistant despite being also genotypically *sacB*⁺: hence they can be used for *sacB*
7 (sucrose) counter-selection in mutagenesis, despite the presence of a *sacB* gene in at least all
8 the sequenced genomes.
9

10 The *sacB* gene from strain 08 was sequenced and compared to the genome sequence of
11 strain K96243, and other sequences that are available in the public domain (Table 1). The
12 genes are all approximately 99% identical at the nucleotide amino acid sequence level (data
13 not shown). Comparison of the sequences of the *sacB* gene from sucrose-sensitive and
14 sucrose-resistant isolates revealed no outstanding differences: There are no frameshift or
15 nonsense codons in the sucrose-resistant class. However, it is known that missense mutation
16 (in cryptic or 'silent' genes) can lead to lack of functional gene product in natural isolates
17 (Innes et al., 2001) and the sucrose-resistant strains have a number of such changes by
18 comparison to sucrose-sensitive strains: isolates 1710a and 1710b and 668 have T->A change
19 at amino acid 23 and isolate Pasteur 52237 has a Q->R change at amino acid residue 259;
20 however, these are conservative substitutions and so may not result in lack of enzyme activity.
21 No changes are detected upstream in the promoter region which correlate to the sucrose
22 phenotype. Thus the reason for the presumed lack of sufficient SacB activity to bring about
23 sucrose-sensitivity is unclear and may be extragenic.
24

25 Since NaCl and temperature are known to influence expression of *sacB* in other bacteria
26 (Kunst and Rapoport, 1995; Abdel-Fattah et al., 2005), we have varied these parameters to
27 determine their effect on the sucrose-sensitivity of single colonies of merodiploid derivatives
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1 of *B. pseudomallei* isolates: sucrose was used at 0, 5 or 10% [w/v]; NaCl was used at 0 or 0.2
2 M; and the temperature was 30 °C, 27 °C or 24 °C. We used strain 08 as a sucrose-resistant
3 isolate and K96243 as a sucrose-sensitive isolate (see above and Table 1). When pDM4,
4 encoding *sacB*, is present in the merodiploid strain derived from 08 (strain JAB1608.1x
5 containing a deleted allele of *pilA*), sucrose sensitivity is observed, even at high cell density
6 (3x10⁶ cfu per plate), and is greater at the lowest temperature. Optimal conditions were found
7 to be LB agar lacking NaCl and containing 10% [w/v] sucrose, with incubation at 24 ° C, for
8 48 hours. We conclude that under these conditions excision of pDM4, and pDM4 derivatives
9 such as pDM4-Tp, should be selected, which is indeed the case: All colonies that were
10 confirmed as sucrose-resistant after purification were also chloramphenicol-sensitive (11/17
11 for JAB1608.1x, over 4 independent experiments), indicating that pDM4 has been excised
12 from the genome. Over many experiments, such clones, for a number of genes, were
13 confirmed as allele replacement mutants by PCR analysis (data not shown).
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31 Since under these same conditions, the endogenous *sacB* in strain K96243 appears to be
32 expressed as evidenced by the lack of growth on sucrose (see Table 1) it would be expected
33 that selection for sucrose-resistant clones from a merodiploid would not be possible.
34 However, notwithstanding the intrinsic sensitivity to sucrose of strain K96243, after 72 hrs,
35 sucrose-resistant colonies of the merodiploid strain JAB16.1x were observed; three out of
36 three colonies in one experiment, under optimised selection conditions, were
37 chloramphenicol-sensitive. PCR analysis confirmed that all three strains had undergone
38 excision of pDM4 with two reverting back to wild-type (Figure 1 A, lanes 4 and 6) and one
39 generating a *pilA* deletion (designated strain K96243Δ*pilA*; Figure 1 A, lane 5).
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54 We conclude that *sacB* counter-selection is possible in *B. pseudomallei*, under appropriate
55 conditions. This is because chromosomal *sacB* expression was minimal (strain 08 and like
56 strains; Table 1); or, in K96243 and like strains, expression from the pDM4-encoded *sacB* is
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1 presumably greater relative to the endogenous *sacB* gene under the optimised conditions,
2 after extended incubation.
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4 To address whether the conditions for sucrose-selection developed for single-gene deletion
5 could be used to generate multi-genic deletions, two merodiploid strains, derived from
6 K96243, designed to delete a number of *Burkholderia pseudomallei* genomic islands (GIs),
7 identified in the annotated K96243 genome sequence (Holden et al., 20004), were tested. A
8 single sucrose-resistant colony derived from strain GI11.1x (a K96243 merodiploid carrying
9 a deleted allele of GII1) was chloramphenicol sensitive and PCR analysis confirmed it had
10 undergone excision of pDM4, but reverting back to the wild-type genotype (data not shown).
11 Two of eight sucrose-resistant colonies derived from strain GI8.1x (a K96243 merodiploid
12 carrying a deleted allele of GI8) were chloramphenicol-sensitive; one of these had excised
13 pDM4, reverting back to wild-type (data not shown), while the other was confirmed to be a
14 deletion strain (designated strain K96243 Δ GI8; Figure 1 B, lanes 3 and 6). Thus, a multi-
15 genic deletion of approximately 92 kbp has been generated in strain K96243. We conclude
16 that *sacB* counter-selection using the conditions described here is effective for the generation
17 of multi-genic deletions.
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39 In conclusion, many routinely used *B. pseudomallei* strains, many whose genomes have
40 been sequenced, are intrinsically sucrose-resistant despite the presence of a *sacB* gene in the
41 genome. The optimised conditions (sucrose 10% [w/v], no NaCl 24 °C) for *sacB* counter-
42 selection allow highly efficient, simple and routine selection of deletion mutants at high cell
43 density in such isolates. Single gene and multigenic deletions were also confirmed for strain
44 K96243, which is sucrose-sensitive, but requires plating at low cell density and extended
45 incubation. Given that it seems likely that *B. pseudomallei* virulence is combinatorial, the
46 widely used *sacB* counter-selection, as described here, allows for the facile generation, in a
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single isolate, of multiple non-polar unmarked deletions thus facilitating the investigation of *B. pseudomallei* virulence.

References

- Abdel-Fattah, A. F., Mahmoud, D. A. R. and Esawy, M. A. T., 2005. Production of levansucrase from *Bacillus subtilis* NRC 33a and enzymatic synthesis of levan and fructo-oligosaccharides. *Curr Microbiol* 51: 402-407.
- Barrett, A. R., Kang, Y., Inamasu, K. S., Son, M. S., Vukovich, J. M. and Hoang, T. T., 2008. Genetic tools for allelic-replacement in *Burkholderia* species. *Appl Environ Microbiol* 74: 4498-4508.
- Brown, N. F. and Beacham, I. R., 2000. Cloning and analysis of genomic differences unique to *Burkholderia pseudomallei* by comparison with *B. thailandensis*. *J Med Microbiol* 49: 993-1001.
- CDC Centres for Disease Control and Prevention. "Melioidosis". www.cdc.gov/nczved/dftbmd/disease_listing/melioidosis_gi.html. Last updated 27 March, 2008.
- Choi, K. H., Mima, T., Casart, Y., Rholl, D., Kumar, A., Beacham, I. R. and Schweizer, H. P., 2008. Genetic tools for select-agent-compliant manipulation of *Burkholderia pseudomallei*. *Appl Environ Microbiol* 74: 1064-1075.
- Currie, B. J., Fisher, D. A., Anstey, N. M. and Jacups, S. P., 2000. Melioidosis: acute and chronic disease, relapse and re-activation. *Trans R Soc Trop Med Hyg* 94: 301-304.
- Dance, D. A., 1991. Melioidosis: the tip of the iceberg? *Clin Microbiol Rev* 4: 52-60.
- Essex-Lopresti, A. E., Boddey, J. A., Thomas, R., Smith, M. P., Hartley, M. G., Atkins, T., Brown, N. F., Tsang, C. H., Peak, I. R. A., Hill, J., Beacham, I. R. and Titball, R. W.,

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2005. A type IV pilin, PilA, contributes to adherence of *Burkholderia pseudomallei* and virulence *in vivo*. *Infect Immun* 73: 1260-1264.

Holden, M. T., Titball, R. W., Peacock, S. J., Cerdeno-Tarraga, A. M., Atkins, T., Crossman, L. C., Pitt, T., Churcher, C., Mungall, K., Bentley, S. D., Sebahia, M., Thomson, N. R., Bason, N., Beacham, I. R., Brooks, K., Brown, K. A., Brown, N. F., Challis, G. L., Cherevach, I., Chillingworth, T., Cronin, A., Crossett, B., Davis, P., DeShazer, D., Feltwell, T., Fraser, A., Hance, Z., Hauser, H., Holroyd, S., Jagels, K., Keith, K. E., Maddison, M., Moule, S., Price, C., Quail, M. A., Rabbinowitsch, E., Rutherford, K., Sanders, M., Simmonds, M., Songsivilai, S., Stevens, K., Tumapa, S., Vesaratchavest, M., Whitehead, S., Yeats, C., Barrell, B. G., Oyston, P. C. and Parkhill, J., 2004. Genomic plasticity of the causative agent of melioidosis, *Burkholderia pseudomallei*. *Proc Natl Acad Sci USA* 101: 14240-14245.

Innes, D., Beacham, I. R., Beven, C. A., Douglas, M., Laird, M. W., Joly, J. C., and Burns, D. M. 2001. The cryptic *ushA* gene (*ushA^c*) in natural isolates of *Salmonella enterica* (serotype Typhimurium) has been inactivated by a single missense mutation. *Microbiology* 147: 1887-1896.

Knodler, L. A., Bestor, A., Ma, C., Hansen-Wester, I., Hensel, M., Vallance, B. A. and Steele-Mortimer, O., 2005. Cloning vectors and fluorescent proteins can significantly inhibit *Salmonella enterica* virulence in both epithelial cells and macrophages: implications for bacterial pathogenesis studies. *Infect Immun* 73: 7027-7031.

Kunst, F. and Rapoport, G., 1995. Salt stress is an environmental signal affecting degradative enzyme synthesis in *Bacillus subtilis*. *J Bacteriol* 177: 2403-2407.

Leelarasamee, A. and Bovornkitti, S., 1989. Melioidosis: review and update. *Reviews of Infectious Disease* 11: 413-425.

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- Milton, D. L. R., O'Toole, P., Horstedt, P and Wolf-Watz, H. 1996. Flagellin is essential for the virulence of *Vibrio anguillarum*. J. Bacteriol. 178: 1310-1319.
- Ried, J. L. and Collmer, A., 1987. An *nptI-sacB* cartridge for constructing directed, unmarked mutations in Gram-negative bacteria by marker exchange- eviction mutagenesis. Gene 57: 239-246.
- Schweizer, H. P., 1992. Allelic exchange in *Pseudomonas aeruginosa* using novel ColE1-type vectors and a family of cassettes containing a portable oriT and the counter-selectable *Bacillus subtilis sacB* marker. Mol Microbiol 6: 1195-1204.
- Sim, S. H., Yu, Y., Lin, C. H., Karuturi, R. K. M., Wuthiekanun, V., Tuanyok, A., Chua, H. H., Ong, C., Paramalingham, S. S., Tan, G., Tng, L., Lau, G., Ooi, E. E., Woods, D., Feil, E., Peacock, S. J., and Tan, P. 2008. The core and accessory genomes of *Burkholderia pseudomallei*: Implications for human melioidosis. PLOS Pathogens 4: e1000178.
- Songsivilai, S. and Dharakul, T., 2000. Multiple replicons constitute the 6.5-megabase genome of *Burkholderia pseudomallei*. Acta Trop 74: 169-179.
- Thongdee, M., Gallagher, L. A., Schell, M. A., Dharakul, T., Songsivilai, S. and Manoil, C., 2008. Targeted mutagenesis of *Burkholderia thailandensis* and *Burkholderia pseudomallei* through natural transformation of PCR fragments. Appl Environ Microbiol. 74: 2985-2989.
- Wiersinga, W. J., van der Poll, T., White, N. J., Day, N. P. and Peacock, S. J., 2006. Melioidosis: insights into the pathogenicity of *Burkholderia pseudomallei*. Nature Reviews 4: 272-282.

1
2 Table 1
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4
5 Growth patterns of *Burkholderia pseudomallei* strains on sucrose-containing media**
6

Naturally sucrose-resistant	Naturally sucrose-sensitive
08	K96243
1026b	576a
1710a*	1655
17a	S13
17b	
307a	
307b	
465a	
668	
Pasteur 52237	

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37 *B. pseudomallei* wild-type strains originated from Australia, Thailand and Singapore and were
38
39 kindly provided by Dr. Tyrone Pitt, Prof. Bart Currie and Dr. Rick Ulrich.

40
41 * 1710b, an isolate from the same patient as 1710a following relapse, was also sucrose-
42
43 resistant.

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46 ** Genome sequence information is available for K96243, S13, 1655, 1710a, 1710b and
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48 Pasteur 52237 (see text).
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Figure legend

Fig. 1. Confirmation of putative deletion strains in *Burkholderia pseudomallei* K96243.

Sucrose selection using media containing no NaCl and 10% [w/v] sucrose is used to generate various deletion strains. **A.** PCR confirms that the three putative strains have undergone excision of pDM4: the expected products are a wild-type allele of 1000 bp and/or a deleted allele of 500 bp. The wild-type and merodiploid contain one or both alleles (lanes 2 and 3, respectively). Two strains are confirmed to have reverted back to wild-type (strains A and C) and one is a *pilA* deletion mutant (strain B; designated K96243 Δ *pilA*). Lanes: **1**, GeneRuler; **2**, K96243; **3**, strain JAB16.1x; **4**, strain A; **5**, strain B; **6**, strain C. **B.** Amplification of genomic DNA from the putative GI8 deletion mutant. Genomic DNA from K96243 and the merodiploid strain (GI8.1x) were used as controls. Only DNA from the merodiploid strain and the deletion strain should amplify a product with the two outer primers (lanes 1-3) and only DNA from K96243 and the merodiploid strain should amplify a product with an upstream and internal primer pair (lanes 4-6). Lanes: **1**, **4**, strain K96243 genomic DNA; **2**, **5**, strain GI8.1x; **3**, **6**, K96243 Δ GI8. All lanes are from the same gel.

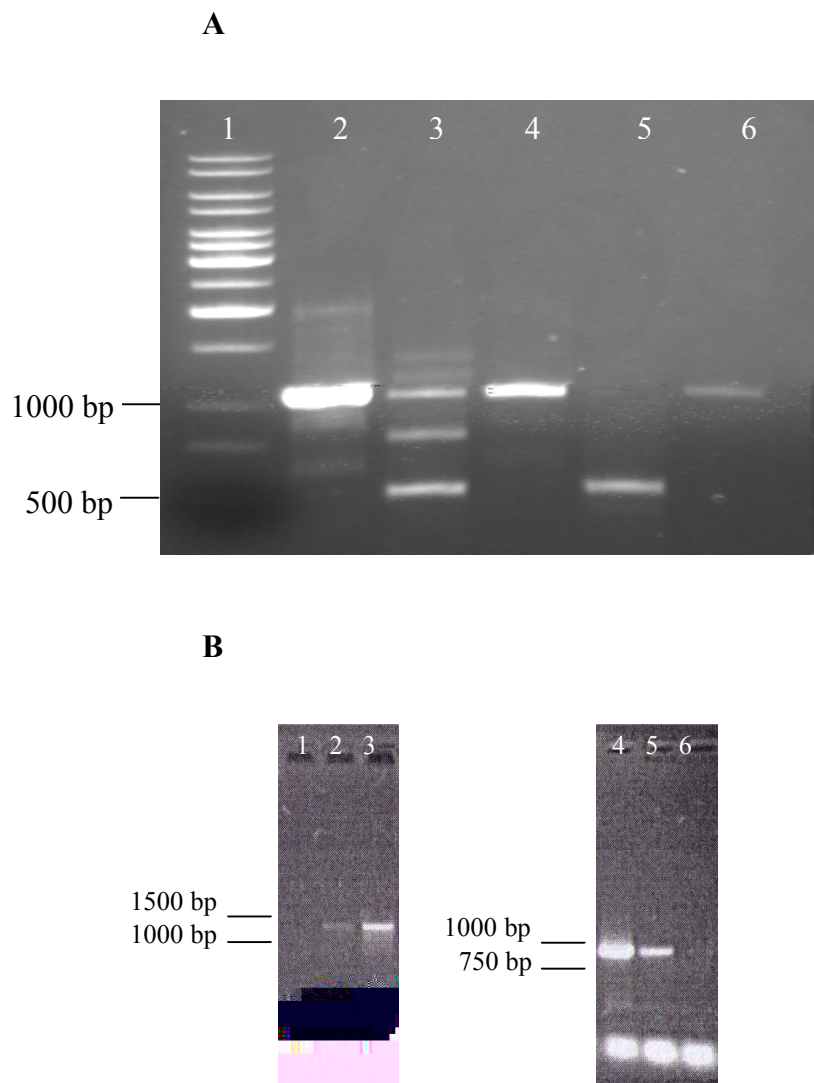


Fig. 1