Applied and Environmental Microbiology, May 2011, p. 3532–3535 Vol. 77, No. 10
0099-2240/11/$12.00 doi:10.1128/AEM.01740-10

Targeted Allele Replacement Mutagenesis of *Corynebacterium pseudotuberculosis*▽†

Caray A. Walker,[1] Willie Donachie,[1] David G. E. Smith,[1,2] and Michael C. Fontaine[1]*

Moredun Research Institute, International Research Centre, Pentlands Science Park, Bush Loan, Penicuik EH26 0PZ, Mid lothian, Scotland, United Kingdom,[1] and Institute of Infection, Inflammation and Immunity, College of Medicine, Veterinary Medicine and Life Sciences, University of Glasgow, 464 Bearsden Road, Glasgow G61 1QH, Scotland, United Kingdom[2]

Received 22 July 2010/Accepted 10 March 2011

A two-step allele replacement mutagenesis procedure, using a conditionally replicating plasmid, was developed to allow the creation of targeted, marker-free mutations in *Corynebacterium pseudotuberculosis*. The relationship between homologous sequence length and recombination frequency was determined, and enhanced plasmid excision was observed due to the rolling-circle replication of the mutagenesis vector. Furthermore, an antibiotic enrichment procedure was applied to improve the recovery of mutants. Subsequently, as proof of concept, a marker-free, *cp40*-deficient mutant of *C. pseudotuberculosis* was constructed.

Corynebacterium pseudotuberculosis is a pathogen of multiple species (1–4, 7, 9–12, 14, 17, 19). To facilitate molecular studies of this organism, we report here an effective allele replacement mutagenesis procedure. A ca. 2.7-kb HpaI/StuI fragment containing the kanamycin resistance gene and 2 open reading frames not associated with plasmid replication (8) was excised from the *Corynebacterium glutamicum* temperature-sensitive (TS) vector, pSFKT2 (16), which replicates via a rolling-circle mechanism (6). A ca. 1.1-kb BamHI fragment from pG$^+$host 9 (15), containing the *ermAM* (an *ermB* derivative) erythromycin resistance gene, was blunt end cloned into pSFKT2. The resulting 5,666-bp plasmid (Fig. 1), designated pCARV (for *C*orynebacterium allele replacement vector), was introduced into *C. pseudotuberculosis* strain 3/99-5 (5) by electroporation as described in the supplemental material. Subsequently, transformed cells were diluted and plated onto brain heart infusion (BHI) agar plates containing 50 ng/ml erythromycin (Erm) prior to incubation for 72 h at 26°C (permissive temperature for plasmid replication). Transformants were unable to grow at 37°C.

The relationship between homologous sequence length and frequency of recombination (chromosomal integration and excision of plasmid) was determined using different size fragments of the *C. pseudotuberculosis cp40* gene (18). All primers used in this study are presented in Table 1. Fragments of *cp40* (224 bp, 432 bp, 750 bp, and 1,020 bp) were amplified by PCR from *C. pseudotuberculosis* 3/99-5 genomic DNA using primer pairs *cp40* F (F for forward) plus *cp40*_0, *cp40* F plus *cp40*_1, *cp40* F plus *cp40*_2, and *cp40* F plus *cp40*_3, respectively. The amplicons from these four fragments were cloned into XmaI-digested pCARV to create recombinant plasmids pCARV001, pCARV002, pCARV003, and pCARV004, respectively.

Aliquots of BHI broth containing 0.05% (vol/vol) Tween 80 (BHIT) plus Erm were inoculated with individual transformants. Cultures were incubated at 26°C to an optical density at 600 nm (OD_{600}) of 0.8 and then at 37°C for 6 to 9 generations (ca. 9 h). The cultures were then diluted and plated onto plates containing BHI agar plus Erm. Plasmid integrants were able to grow at 37°C. The integration frequency of each construct was expressed as the erythromycin-resistant proportion of the total cell population. Triplicate experiments revealed that plasmid integration increased linearly with increasing length of homologous sequences, with average integration frequencies of 1.81×10^{-1}, 2.23×10^{-1}, 2.80×10^{-1}, and 4.75×10^{-1} for pCARV001, pCARV002, pCARV003, and pCARV004, respectively.

To assess plasmid excision, aliquots of BHIT broth plus Erm were inoculated with plasmid integrants. Cultures were incubated at 37°C until stationary phase, then diluted 10^{-5} in BHIT broth, and incubated at 26°C to stationary phase. Bacteria were recovered on BHI agar plates at 37°C, and then single colonies were replica plated onto BHI agar plates with or without Erm. After incubation at 37°C, colonies unable to grow on plates containing Erm had undergone plasmid excision. Excision rates increased with increasing length of homologous sequence. However, variability was observed between experiments (Table 2), and the frequency of secondary recombination (excision) events was higher than anticipated. Previous studies have assigned high excision rates to the single-stranded DNA intermediates generated during rolling-circle-type replication (13).

Recombination decreased with shorter homologous sequence lengths, so the bacteriostatic action of erythromycin was assessed as a means of enriching cultures. Immediately after plasmid excision, cultures were diluted into BHIT broth plus Erm and incubated at 37°C briefly to allow growth of resistant cells (with concurrent inhibition of the growth of nonresistant cells). The cultures were then supplemented with ampicillin (to 100 μg/ml) and monitored until the OD_{600} had dropped (indicating cell lysis) and become static. Subsequently, the cells were washed and diluted, and diluted cells were replica plated onto BHI agar plates with and without

* Corresponding author. Mailing address: Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik EH26 0PZ, Mid Lothian, Scotland, United Kingdom. Phone: 44 0131 443 5111, ext. 440. E-mail: michael.fontaine@moredun.ac.uk.

† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 18 March 2011.

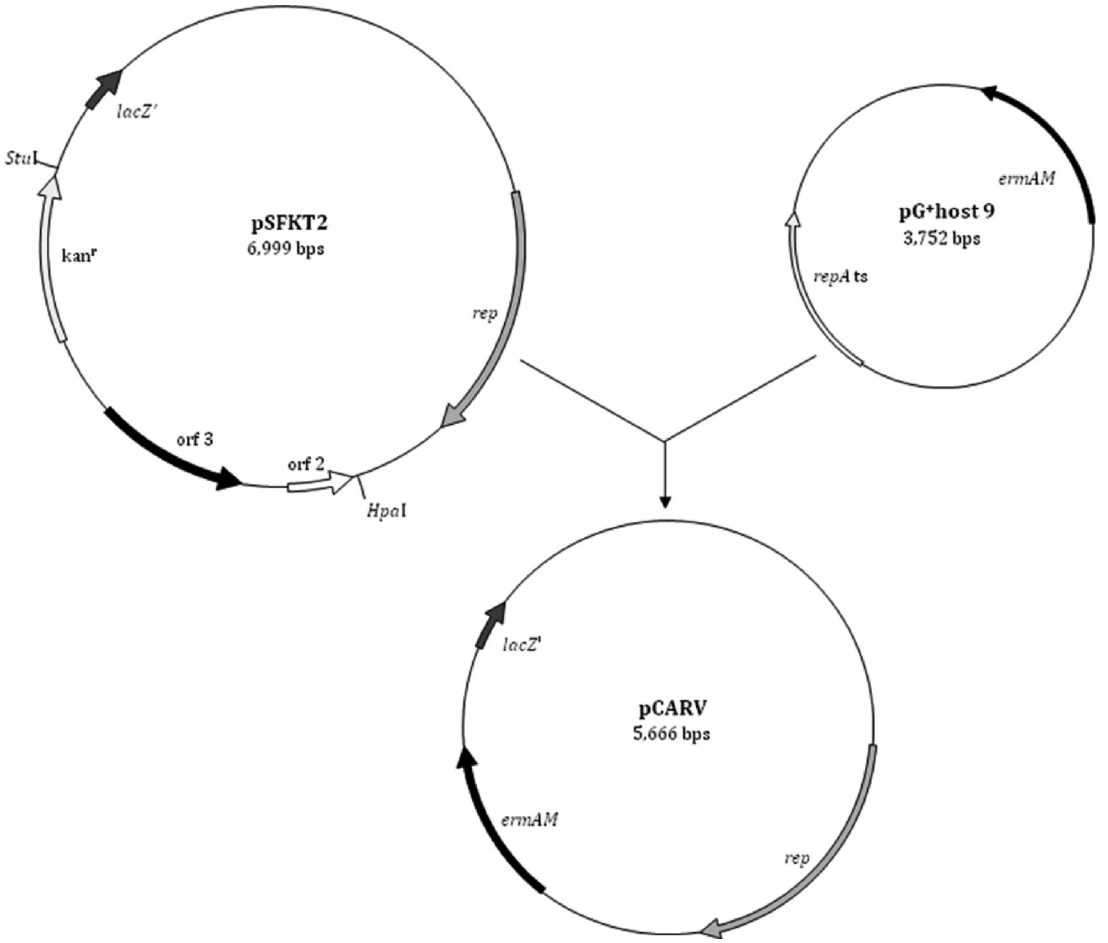

FIG. 1. Cloning strategy for the construction of pCARV.

erythromycin. The number of colonies derived from cells that had undergone plasmid excision was then expressed as a percentage of the total bacterial population (Table 2). Ampicillin enrichment increased recovery of the desired cell population and was most evident for the shortest *cp40* fragment, where cells that had lost pCARV001 increased from a maximum of 13% without enrichment to 73% following enrichment.

TABLE 1. PCR and sequencing primers used in this study

Primer	Sequence (5′–3′)[a]	Target[b]
cp40 F	CCCGGGGCAACAGTGACCCCCAAGGGA (A)	bp 1135–1155
*cp40*_0	CCCGGGAGAGCGGAAGTTATGCCTA (A)	bp 1403–1385
*cp40*_1	CCCGGGCTGGAGCTTGTCTTTCCAA (A)	bp 1608–1590
*cp40*_2	CCCGGGCGGAGTTCCATGTCTACG (C)	bp 1916–1899
*cp40*_3	CCCGGGCTGGCAGGAATTGATCTTG (A)	bp 2199–2181
*cp40*_SCO_1	CCTTCAGTACCCGGATTAGC	bp 114–133
*cp40*_SCO_2	GAAATCACAGACCCCTGC	bp 3573–3556
cpAE1	GTCGACGCGGACTTTGTAAAGTTTGC (B)	bp 332–351
cpAE2	CTCGAGGCGTGAGACTGATCGAGG (C)	bp 1374–1357
cpAE3	CTCGAGTTCACCACACTCAAACCGAC (C)	bp 2380–2399
cpAE4	GTCGACGACCTTTTTGTTACCGTGC (B)	bp 3420–3402
Δ*cp40* DCO_F	CTTGCCCAGGATTAAATGC	bp 1272–1290
Δ*cp40* DCO_R	CGCCCGTGAGATTATTTTT	bp 2571–2553
*cp40*_RT_F	CTCGATCAGTCTCACGC	bp 1358–1374
*cp40*_RT_R	CTGTAGACGATTCGCCG	bp 2455–2439
*cp40*_seq_F	CGGCAACATCTAGCTGC	bp 1088–1104
*cp40*_seq_R	GCTAAAACAAAACGGCG	bp 2739–2723
pCARV_MCS_1	ACACAGGAAACAGCTATGACC	pCARV
pCARV_MCS_2	AAACGACGGCCAGTGAAT	pCARV

[a] Restriction endonuclease recognition sites are underlined. The specific restriction endonuclease recognition site is indicated by a capital letter in parentheses after the sequence as follows: A, XmaI; B, SalI, and C, XhoI.

[b] Unless otherwise specified, all primers are specific for GenBank accession number JF299259.

TABLE 2. Excision rates of pCARV-based constructs

Plasmid	Size of insert (bp)	Excision rate range (%)	
		Without enrichment	With enrichment
pCARV001	224	0–13	25–73
pCARV002	432	0–50	43–97
pCARV003	750	2–54	45–89
pCARV004	1,020	23–69	48–81

A *cp40*-deficient mutant of *C. pseudotuberculosis* was then created. PCR amplification of the 5′ and 3′ chromosomal regions flanking the gene was conducted using primer pairs cpAE1 plus cpAE2 (fragment 1) and cpAE3 plus cpAE4 (fragment 2). Fragment 1 comprised 1,032 bp of upstream flanking sequence, including the first 30 bp of *cp40*, while fragment 2 comprised 919 bp of downstream flanking sequence, containing the last 105 bp of *cp40*. The two amplicons were digested with XhoI and ligated, and the ligation product was further amplified by PCR using primer pair cpAE1 plus cpAE4. The resulting ca. 2.1-kb fragment (designated Δ*cp40* fragment), containing an in-frame deletion of 1,041 bp, was then cloned into SalI-digested pCARV to create pCARV005.

Chromosomal integration of the pCARV005 plasmid was conducted (Fig. 2), following which 10 plasmid integrants were further analyzed. Recombination between homologous sequences resulted in different integration structures depending on whether this occurred at the 5′ or 3′ end of the Δ*cp40* fragment (Fig. 3). To determine the ratio of 5′-end recombination/3′-end recombination, 2 PCRs were conducted for each integrant using primer pairs pCARV_MCS_1 (MCS for multiple-cloning site) plus *cp40*_SCO_1 (SCO for single crossover) (upstream PCR) and pCARV_MCS_2 plus *cp40*_SCO_2 (downstream PCR). Recombination at the 5′ end of the Δ*cp40* fragment resulted in fragments of 2,364 bp for the upstream PCR and 3,295 bp for the downstream PCR, while 3′-end recombination resulted in fragments of 3,399 bp for the upstream PCR and 2,259 bp for the downstream PCR. Analyses revealed that recombination at the 5′ end or 3′ end of the Δ*cp40* fragment occurred at a ratio of 60:40 (data not shown).

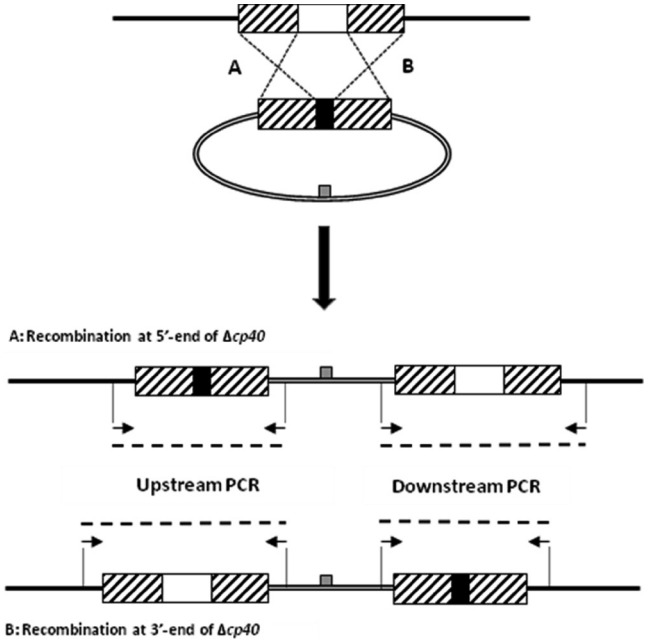

FIG. 2. Integration of pCARV005 into the *C. pseudotuberculosis* chromosome was driven by recombination between homologous sequences (hatched bars) flanking the wild-type (white bar) and mutant (black bar) *cp40* alleles. The plasmid-encoded erythromycin gene (small gray square) allowed isolation of integrants at 37°C. Recombination at either the 5′ or 3′ end of each allele (A or B) affected the structure of the resulting plasmid integrants, which were elucidated by PCR with primers (arrows) directed to chromosomal sequence (solid black line) and vector sequence (gray line).

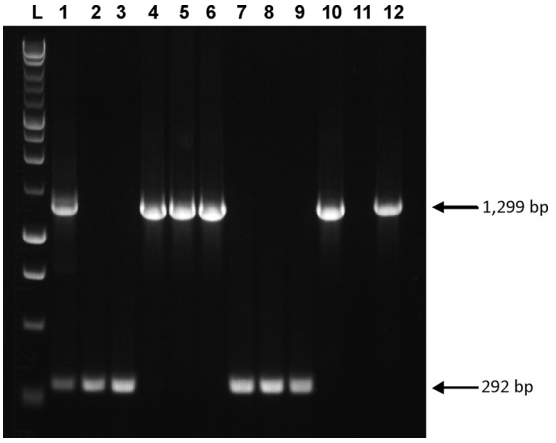

FIG. 3. PCR analysis of *C. pseudotuberculosis* secondary-crossover mutants. Lane L, 1-kb ladder (lane L); lane 1, plasmid integrant; lanes 2, 3, 7, 8, and 9, Δ*cp40* mutant; lanes 4, 5, 6, and 10, wild-type *cp40*; lane 12, *C. pseudotuberculosis* wild-type control.

Excision of pCARV005 and ampicillin enrichment were conducted, and 10 isolates were further analyzed to determine whether the two-step recombination process had left wild-type or mutant *cp40* in the chromosome (depending on whether primary and secondary recombination events had occurred at the same or different ends of *cp40*). PCR analysis of genomic DNA was conducted (Fig. 3) using primer pair Δ*cp40* DCO_F (DCO for double crossover) plus Δ*cp40* DCO_R (R for reverse) (a 1,299-bp or 292-bp product would be amplified from isolates containing the *cp40* gene or Δ*cp40* fragment, respectively). One isolate retained chromosomally integrated pCARV005, determined by amplification of 1,299-bp and 292-bp bands; this isolate was subsequently found to be Erm resistant and had been chosen in error. Of the remaining isolates, 4 retained *cp40*, while 5 possessed the Δ*cp40* fragment.

The chromosomal region spanning Δ*cp40* in the 5 mutants was PCR amplified with primer pair *cp40*_seq_F plus *cp40*_seq_R and sequenced in both directions using the same primers. For each mutant, the sequence was as expected (data not shown). One mutant was designated 3/99-5_Δ*cp40*, and reverse transcription-PCR (RT-PCR) analysis of mRNA from log-phase cultures of this mutant and the wild-type parent was conducted using primer pair *cp40*_RT_F plus *cp40*_RT_R. A full-length transcript of ca. 1.14 kb was amplified from the wild-type strain, while an amplicon of ca. 0.14 kb was amplified

from strain 3/99-5_Δ*cp40* (data not shown). The allele replacement system presented here therefore offers the means to mutate specific *C. pseudotuberculosis* genes, with a high level of efficiency, and without requiring the use of chromosomally located selectable markers.

Nucleotide sequence accession number. The nucleotide sequence of the *C. pseudotuberculosis* chromosomal region containing the *cp40* gene has been deposited in GenBank under accession number JF299259.

We are grateful to J. Nakamura for providing pSFKT2 and to E. Maguin for providing pG$^+$host 9.

We also acknowledge the Scottish Government Rural and Environment Research Analysis Directorate (RERAD) and the Moredun Foundation (registered charity SC022515) for funding this work.

REFERENCES

1. **Addo, P. B., and F. I. Eid.** 1978. Caseous lymphadenitis of sheep and goats in Nigeria. Bull. Anim. Health Prod. Afr. **26:**37–41.
2. **Baird, G., B. Synge, D. Armstrong, and D. Dercksen.** 2001. Caseous lymphadenitis in sheep in the United Kingdom. Vet. Rec. **149:**399.
3. **Batey, R. G.** 1986. Frequency and consequence of caseous lymphadenitis in sheep and lambs slaughtered at a Western Australian abattoir. Am. J. Vet. Res. **47:**482–485.
4. **Batey, R. G.** 1986. Pathogenesis of caseous lymphadenitis in sheep and goats. Aust. Vet. J. **63:**269–272.
5. **Connor, K. M., M. M. Quirie, G. Baird, and W. Donachie.** 2000. Characterization of United Kingdom isolates of *Corynebacterium pseudotuberculosis* using pulsed-field gel electrophoresis. J. Clin. Microbiol. **38:**2633–2637.
6. **Fernandez-Gonzalez, C., R. F. Cadenas, M. F. Noirot-Gros, J. F. Martin, and J. A. Gil.** 1994. Characterization of a region of plasmid pBL1 of *Brevibacterium lactofermentum* involved in replication via the rolling circle model. J. Bacteriol. **176:**3154–3161.
7. **Goldberger, A. C., B. A. Lipsky, and J. J. Plorde.** 1981. Suppurative granulomatous lymphadenitis caused by *Corynebacterium ovis* (*pseudotuberculosis*). Am. J. Clin. Pathol. **76:**486–490.
8. **Goyal, D., et al.** 1996. A cryptic plasmid pBL1 from *Brevibacterium lactofermentum* causes growth inhibition and filamentation in *Escherichia coli*. Plasmid **36:**62–66.
9. **Hemond, V., S. Rosenstingl, M. L. Auriault, M. J. Galanti, and M. Gatfosse.** 2009. Axillary lymphadenitis due to *Corynebacterium pseudotuberculosis* in a 63-year-old patient. Med. Mal. Infect. **39:**136–139. (In French.)
10. **Hughes, J. P., and E. L. Biberstein.** 1959. Chronic equine absesses associated with *Corynebacterium pseudotuberculosis*. J. Am. Vet. Med. Assoc. **135:**559–562.
11. **Join-Lambert, O. F., et al.** 2006. *Corynebacterium pseudotuberculosis* necrotizing lymphadenitis in a twelve-year-old patient. Pediatr. Infect. Dis. J. **25:**848–851.
12. **Keslin, M. H., E. L. McCoy, J. J. McCusker, and J. S. Lutch.** 1979. *Corynebacterium pseudotuberculosis*. A new cause of infectious and eosinophilic pneumonia. Am. J. Med. **67:**228–231.
13. **Leenhouts, K. J., J. Kok, and G. Venema.** 1991. Replacement recombination in *Lactococcus lactis*. J. Bacteriol. **173:**4794–4798.
14. **Lopez, J. F., F. M. Wong, and J. Quesada.** 1966. *Corynebacterium pseudotuberculosis*. First case of human infection. Am. J. Clin. Pathol. **46:**562–567.
15. **Maguin, E., H. Prevost, S. D. Ehrlich, and A. Gruss.** 1996. Efficient insertional mutagenesis in lactococci and other gram-positive bacteria. J. Bacteriol. **178:**931–935.
16. **Nakamura, J., et al.** 2006. Temperature-sensitive cloning vector for *Corynebacterium glutamicum*. Plasmid **56:**179–186.
17. **Shpigel, N. Y., D. Elad, I. Yeruham, M. Winkler, and A. Saran.** 1993. An outbreak of *Corynebacterium pseudotuberculosis* infection in an Israeli dairy herd. Vet. Rec. **133:**89–94.
18. **Wilson, M. J., M. R. Brandon, and J. Walker.** 1995. Molecular and biochemical characterization of a protective 40-kilodalton antigen from *Corynebacterium pseudotuberculosis*. Infect. Immun. **63:**206–211.
19. **Yeruham, I., et al.** 2004. A herd level analysis of a *Corynebacterium pseudotuberculosis* outbreak in a dairy cattle herd. Vet. Dermatol. **15:**315–320.