Disruption of *adhC* reveals a large duplication in the *Mycobacterium smegmatis* mc²155 genome

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Disruption of the *adhC* gene of *Mycobacterium smegmatis* mc²155, by standard gene replacement methods, revealed that there are two copies of this gene within a large duplication of the *M. smegmatis* mc²155 genome. *M. smegmatis* AdhC^{+/-} and *M. smegmatis* AdhC^{-/-} mutants were obtained when one or two *adhC* copies, respectively, were disrupted by homologous recombination. Southern blot analysis of *Dra*I restriction digests of the DNA from these mutants and from wild-type *M. smegmatis* mc²155, resolved by PFGE, showed that the duplication size may be at least ~ 250 kb. The single and double knockout mutants were characterized and compared with the *M. smegmatis* wild-type. A growth disadvantage and a different morphology were associated with the loss of expression of one or both of the *adhC* copies, but both mutants were still acid-fast. Findings in this study indicate that the process of chromosomal duplication in *M. smegmatis* is ongoing and remains a potent source of genome dynamics. Hence, the *M. smegmatis* mc²155 genome might be larger than previously thought.

Keywords: homologous recombination, genomic duplication, merodiploidy, alcohol dehydrogenase C

INTRODUCTION

Tuberculosis remains a major world health problem and is responsible for approximately 2 million deaths each year (Dye *et al.*, 1999). Although this infection can be treated effectively with multidrug therapy, incomplete treatment has led to the development of drug-resistant strains leading to the urgent need for specific targets for new anti-microbial agents. Since the cell wall is an attractive target for antibiotic development, considerable efforts have been focused on discovering the metabolic steps that are essential for biosynthesis of mycobacterial cell-wall components (Baulard *et al.*, 1999; Besra *et al.*, 1997; Lee *et al.*, 1996; Mikusova *et al.*, 1996).

Alcohol dehydrogenase C (ADHC) has been suggested to be a potential target for the development of new

antibiotics. This protein, initially purified and biochemically characterized from *Mycobacterium bovis* BCG, was suggested to contribute to the hydrophobic content of the mycobacterial envelope through its involvement in the biosynthesis of the free lipids required for envelope formation (De Bruyn *et al.*, 1981a, b; Wilkin *et al.*, 1999). The *M. bovis* BCG ADHC (BCG-ADHC) gene was cloned and found to be identical to the *adhC* gene from *M. tuberculosis* (Stelandre *et al.*, 1992; Wilkin *et al.*, 1999). BCG-ADHC is a dimeric zinc, NADP-dependent enzyme belonging to the long-chain alcohol/polyol dehydrogenase family, class C. To test the hypothesis that ADHC is essential in mycobacterial envelope formation we undertook experiments to knock out the *adhC* gene and examine the effects of this mutation.

To avoid the difficulty of generating mutants by homologous recombination in slow-growing mycobacterial strains (McFadden, 1996), and to speed up the clarification of the physiological role of mycobacterial ADHCs, we first looked for such an enzyme in a fast-growing and non-pathogenic mycobacterial strain. We

Abbreviations: ADHC, alcohol dehydrogenase C; BCG-ADHC, *M. bovis* BCG ADHC; DCO, double cross-over; Ms-ADHC, *M. smegmatis* mc²155 ADHC; SCO, single cross-over.

have previously reported the identification of *M. smegmatis* mc²155 ADHC (Ms-ADHC; Galamba *et al.*, 2001), which shares 78 % identity with BCG-ADHC and *M. tuberculosis* ADHC. The Ms-ADHC gene was cloned and sequenced, and the protein was purified, partially characterized and compared with BCG-ADHC. The two enzymes were found to be similar and functioned as aldehyde reductases *in vitro*, processing alcohols far less efficiently than aliphatic and aromatic aldehydes. It was also found that Ms-ADHC shares a strong degree of amino acid sequence similarity with the ADHCs of *M. avium* and *M. paratuberculosis* (76 %), and *M. leprae* (75 %). These results suggested that *M. smegmatis* mc²155 could be used as a model to study the physiological role of the alcohol dehydrogenases in pathogenic mycobacteria. Thus, we used the cloned *adhC* gene to generate an ADHC knockout mutant of *M. smegmatis* mc²155, by homologous recombination with a double cross-over (DCO) event. The resulting *adhC*-disrupted mutant should allow us to test the potential role of ADHCs in the mycobacterial cell envelope and to evaluate their *in vivo* significance as a target for anti-tuberculosis drugs.

In this paper we describe how the experiments undertaken to disrupt the *adhC* chromosomal allele (with a kanamycin-resistance gene) uncovered a large duplication in the *M. smegmatis* mc²155 genome. In addition, the *AdhC*^{+/-} and *AdhC*^{-/-} mutants were characterized and compared to the *M. smegmatis* wild-type with respect to growth and the utilization of some aldehydes *in vitro*.

METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are detailed in Table 1. *Escherichia coli* DH5 α was cultured on Luria-Bertani (LB; Sambrook *et al.*, 1989) agar or in LB broth. For *in vitro* growth comparison studies the *M. smegmatis* strains were cultured in Middlebrook 7H9 broth or on Middlebrook 7H10 supplemented with 10 % (v/v) oleic acid-albumin complex for 36 h at 37 °C Becton Dickinson.

Construction of the suicide delivery vector pAGA6KM. DNA manipulations were carried out according to standard techniques (Sambrook *et al.*, 1989). The cloning strategy used for generating the suicide delivery vector is illustrated in Fig. 1. Primers G381, 5'-CGCGGATCCTCTCACAGGGTGTTC-3', and G382, 5'-CGCGGATCCGTTCCGGTGGTGATGCCGAA-3', (*Bam*HI recognition sequences are underlined), were used to amplify a 1951 bp fragment from pAGA5 (Galamba *et al.*, 2001). The resulting PCR product was cloned into the *Bam*HI site of pUC19 to generate pAGA6. A cassette containing the *aphA-3* gene, which confers resistance to kanamycin (Menard *et al.*, 1993), was used to disrupt the *adhC* gene. This cassette should not create polar mutations in genes downstream of *adhC*, and was obtained by PCR from pUC18K, using the primers G379, 5'-GTGAATTCGACGCGCCGCCCGGGTG-3', and G380, 5'-GACTCTAGAGGCGGCCCGGTCATTAT-3', (*Not*I recognition sequences are underlined). The final delivery vector pAGA6KM contained the following selectable markers: a kanamycin-resistance gene; a hygromycin-resistance gene;

and a gene that confers sucrose sensitivity. pAGA6KM also lacked a mycobacterial origin of replication (*oriM*); therefore it was a suicide plasmid unable to replicate in mycobacteria.

Electroporation of *M. smegmatis*. *M. smegmatis* mc²155 was cultured in supplemented nutrient broth [SNB; nutrient broth plus 0.2 % (v/v) glycerol and 0.05 % (w/v) Tween 80], in a shaking incubator at 37 °C and 150 r.p.m. When OD₆₀₀ 0.6 was reached, cells were kept on ice for 2 h and then harvested by centrifugation (11000 g, 10 min at 4 °C). The cell pellet was then washed in cold 10 % (w/v) glycerol (1/6 vol. nutrient broth) and centrifuged (4000 g, 10 min at 4 °C). The cell pellet was washed twice more with cold 10 % (w/v) glycerol (1/15 and 1/30 vol. SNB, respectively), resuspended in 2 ml 10 % (w/v) glycerol and stored at -70 °C until needed. Plasmid DNA (100 ng) was pretreated with 100 mJ UV light cm⁻² (Hinds *et al.*, 1999) and used to electroporate 25 μ l of *M. smegmatis* competent cells in a 0.1 cm gap electroporation cuvette (Bio-Rad) at 1.66 kV, 26 μ F and 200 Ω using a Gene Pulser (Bio-Rad). Cells were immediately diluted with 1 ml fresh SNB at room temperature, incubated in a shaking incubator for 3 h at 37 °C and 150 r.p.m. and plated onto supplemented nutrient broth agar [SNBA; nutrient broth agar plus 0.2 % (v/v) glycerol] containing 20 μ g kanamycin ml⁻¹.

Analysis of the transformants. To distinguish between the single cross-over (SCO) and DCO homologous recombinants, the Kan^R clones were plated out onto both sucrose/kanamycin SNBA [10 % (w/v) sucrose; 20 μ g kanamycin ml⁻¹] and hygromycin SNBA (100 μ g hygromycin ml⁻¹) plates. The Kan^R Hyg^S Suc^R DCOs were further analysed by Southern blotting and PCR. The DNA from the *M. smegmatis* mutants was extracted as previously described (Galamba *et al.*, 2001). Southern blots of the DNA from each mutant were prepared after the DNA had been digested with *Bam*HI, *Apal*, *Sfi*I or *Bgl*II. The restriction fragments were run out on 1 % agarose gels and transferred to Hybond-N nylon membranes. Hybridizations were performed at 65 °C as previously described (Galamba *et al.*, 2001); the probes used for the hybridizations were *Padh*, *Pkan*, *PscB* and *Pdws* (see Fig. 1 and Table 1). All of the probes were labelled by random priming with [α -³²P]dCTP using a random priming kit (Amersham). PCR analysis was carried out either on extracted DNA or on individual colonies from the SCO and DCO recombinant strains. To carry out PCR from individual colonies, a colony from each transformant was resuspended in 1 ml water, boiled for 25 min to liberate chromosomal DNA and cooled on ice for 10 min. The liberated DNA was then used as a template for PCR amplification. PCR was carried out in 50 μ l reaction volumes containing 15 μ l of colony suspension and *Taq* DNA polymerase (Promega) under the following conditions: 1 min at 95 °C; 30 cycles of 1 min at 95 °C, 1 min at 60 °C, 1.5 min at 72 °C; and, a final 10 min extension period at 72 °C. Primers R59, 5'-TTCGTCGACTCCTGCCGAGA-3', and G345, 5'-GTCGATGACGAAGCGGTAGCGCAC-3', were used for the PCR amplification. These primers were designed to amplify a 760 bp fragment from the wild-type strain, and a 1500 bp fragment from mutant strains that contained the *aphA-3* cassette insertion.

PFGE and Southern blot hybridization of *M. smegmatis* wild-type and mutant strains. Chromosomal DNA embedded in agarose plugs was obtained from *M. smegmatis* mc²155, *M. smegmatis* *AdhC*^{+/-} and *M. smegmatis* *AdhC*^{-/-} grown in 5 ml modified Middlebrook 7H9 broth (Difco) containing 0.5 M sucrose, 0.05 % (w/v) Tween 80, 0.2 % (w/v) D-glucose and 10 % (v/v) oleic acid-albumin complex (Beckman Dickson), for 24 h at 37 °C. Four hundred microlitres of a solution

Table 1. Bacterial strains, plasmids and probes used in this study

Strain/plasmid/probe	Relevant characteristics	Source/reference*
Bacteria		
<i>E. coli</i> DH5 α	Competent cells for cloning and plasmid propagation	Gibco-BRL
<i>M. smegmatis</i> mc ² 155	Wild-type	Galamba <i>et al.</i> (2001)
<i>M. smegmatis</i> AdhC ^{+/-}	Single knockout mutant of mc ² 155	This study
<i>M. smegmatis</i> AdhC ^{-/-}	Double knockout mutant of mc ² 155	This study
Plasmids		
pUC19	Vector	Gibco-BRL
pAGA5	Contains a 3.3 kb fragment including <i>adhC</i> from <i>M. smegmatis</i> mc ² 155	Galamba <i>et al.</i> (2001)
pAGA6	<i>amp</i> , 1951 bp fragment containing coding sequence of <i>adhC</i> with an internal <i>NotI</i> site, and flanking regions 475 bp and 429 bp either side of <i>adhC</i>	This study
pUC18K	Contains an <i>aphA-3</i> cassette with an internal <i>DraI</i> site; Kan ^R	R. Menard; Menard <i>et al.</i> (1993)
pAGA6K	<i>amp</i> , <i>adhC::aphA-3</i> ; Kan ^R	This study
pGOAL19	<i>hyg</i> , P _{Ag 85} - <i>lacZ</i> , P _{hsp 60} - <i>sacB</i> ; Hyg ^R Suc ^S	T. Parish & N. Stoker; Parish & Stoker (2000)
pAGA6KM	<i>hyg</i> , <i>adhC::aphA-3</i> , P _{hsp 60} - <i>sacB</i> ; Kan ^R Hyg ^R Suc ^S . Lacks a mycobacterial origin of replication (<i>oriM</i> ⁻), therefore is a suicide plasmid	This study
Probes		
Padh	A 1951 bp fragment containing the <i>adhC</i> gene coding sequence obtained by PCR amplification of pAGA5 with primers G381 and G382	This study
Pkan	The <i>aphA-3</i> cassette	This study
PsacB	A 2699 bp fragment containing the <i>sacB</i> gene obtained by the digestion of pGOAL19 with <i>PacI</i> and <i>BamHI</i>	This study
Pdws	A 1230 bp <i>XmnI</i> - <i>KpnI</i> fragment downstream of the ADHC ORF obtained by digestion of pAGA5	This study
P600	A 760 bp fragment of the ADHC ORF downstream of where <i>adhC::aphA-3</i> was inserted; obtained from pAGA5	This study
Pups	A 343 bp <i>BamHI</i> - <i>EcoRV</i> fragment upstream of the ADHC ORF	This study

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containing 0.2 M glycine, 60 μ g D-cycloserine ml⁻¹, 20 mM LiCl, 200 μ g lysozyme ml⁻¹ and 5 mM EDTA were then added to the cultures; the cultures were incubated for an additional 16 h and were then centrifuged at 1000 g for 20 min at 4 °C. The bacteria were recovered and resuspended in TS buffer (50 mM Tris, 0.5 M sucrose, pH 7.6) (1/50 culture vol.). The cell suspensions were transferred to microcentrifuge tubes, immediately frozen in dry ice-methanol and then thawed on wet ice. The cell suspensions were then mixed with an equal volume of 1% low-melting-point agarose at 50 °C (Incert agarose; FMC Bioproducts) in TEN buffer (50 mM Tris, 250 mM EDTA, 200 mM NaCl, pH 7.6) and cast in plugs. The plugs were left to set for 20 min, and were incubated overnight at 37 °C in TE buffer (10 mM Tris, 1 mM EDTA, pH 8) containing 1 mg lysozyme ml⁻¹ to lyse the cells. The plugs were then transferred to TE buffer containing 1% SDS and 1 mg proteinase K ml⁻¹ and incubated for 48 h, 55 °C. The plugs were transferred into fresh TE buffer and washed for 30 min, then transferred to TE buffer containing 0.04 mg PMSF ml⁻¹ incubated at 55 °C for 30 min. They were then washed three times, using only fresh TE buffer for each wash. The

plugs were stored in 0.2 M EDTA at 4 °C. Before restriction digestion the plugs were washed twice in TE/Triton X-100 (0.1%) (v/v) at 4 °C for 1 h, and twice in restriction enzyme buffer/Triton X-100 (0.1%) (v/v) for 1 h at room temperature; the plugs were then incubated overnight at 37 °C in the presence of *DraI* (20 U). The restriction fragments were separated on a 1% agarose gel (Seakem GTG, FMC) in 0.5 × TBE buffer (0.025 M Tris, 0.5 mM EDTA and 0.025 M boric acid) containing 50 μ M thiourea, using a CHEF Mapper system (Bio-Rad) at 14 °C and 200 V. For *DraI*-digested DNA from the wild-type and from clone 31, pulse times were ramped linearly from 1–40 s for 22 h; pulse times for *DraI*-digested DNA from clone 18 were ramped 5–20 s for 20 h. Bacteriophage λ concatemers (48.5 kb) were used as DNA standards. Gels were stained with ethidium bromide, and examined and photographed under UV light on a trans-illuminator. The agarose gels were then treated with 0.25 M HCl for 30 min before being treated following the standard Southern method and transferred by capillarity to a Hybond-N nylon membrane. Southern blot hybridizations were performed using probes P600 and Pups (Table 1). P600 was

Fig. 1. Cloning strategy for generating the pAGA6KM suicide delivery vector. A 1951 bp fragment, containing the *M. smegmatis* *adhC* gene, was obtained from pAGA5 by PCR and was cloned into the unique *Bam*HI site of pUC19, to generate pAGA6. An 873 bp kanamycin cassette (*aphA-3*), obtained from pUC18K by PCR, was cloned into the unique *Not*I site of the *adhC* in pAGA6, to give pAGA6K. The suicide delivery vector pAGA6KM was generated by replacing the 3026 bp *Bam*HI *lacZ* fragment of pGOAL19 with the 2.8 kb *Bam*HI fragment of pAGA6K which contained the disrupted *adhC* (*adhC::aphA-3*).

obtained by PCR amplification of pAGA5 with the primers G344, 5'-GCGCCGCTGTTGTGCGCGGC-3', and G345.

SDS-PAGE and Western blot analysis. SDS-PAGE was carried out as described by Laemmli (1970) with 12 % polyacrylamide gels on a Miniprotean II system (Bio-Rad). Proteins were transferred onto nitrocellulose filters with an LKB Multiphor II electrophoresis unit by semidry electroblotting. ADH was detected by incubation with a murine monoclonal antibody

raised against the BCG-ADHC (4B5) (Stelandre *et al.*, 1992) and developed with the Protoblot Western blot alkaline phosphatase system (Promega), according to the manufacturer's instructions.

Biochemical characterization of the *adhC* knockout mutants.

M. smegmatis mc²155, AdhC^{+/−} and AdhC^{−/−} mutants were grown on Sauton medium as surface pellicles for 3 d at 37 °C. The pellicles were recovered by filtration, sonicated with a

probe tip sonicator (Vibra Cell) at 50 % duty cycle for 10 min and then centrifuged (11000 g, 15 min). The protein concentration in the cell lysates was determined by the Coomassie brilliant blue method (Spector, 1978) with BSA as the standard. ADH activity was assayed by monitoring the oxidation of NADPH by using a spectrophotometer (DU640B; Beckman Coulter) at A_{340} (A_{366} for cinnamaldehyde). This assay was carried out at room temperature in a 1 ml reaction mixture [(0.02 M $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer, pH 7.3; 0.25 mM NADPH; 25 µg total protein) plus the aldehyde substrate (either 100 µM octanal, 200 µM benzaldehyde, 200 µM cinnamaldehyde or 1 mM butyraldehyde)].

RESULTS

Homologous recombination at the *adhC* locus of *M. smegmatis*

To generate an *M. smegmatis* AdhC⁻ mutant by gene replacement, the *adhC* coding region was interrupted at its unique *NotI* site by insertion of a kanamycin-resistance marker gene (*aphA-3*). A one-step strategy (direct screening of DCOs) using the suicide vector pAGA6KM was used (Fig. 1). Plasmid DNA was pretreated with UV light to enhance the recombination frequency (Hinds *et al.*, 1999) and used to electroporate *M. smegmatis* competent cells. To enable the selection of *M. smegmatis* cells that had undergone gene replacement events and reduce the number of colonies to be tested for DCOs, we used *sacB* and *hyg* as screening markers. Gene replacement events with looping-out of intervening plasmid sequences result in the loss of *sacB* and *hyg* and render the organism resistant to sucrose and sensitive to hygromycin again. When used individually there are often problems with both of these markers. Spontaneous Hyg^R mutant clones can be obtained (Parish *et al.*, 1999), and though the *sacB* gene has previously been used for negative selection in mycobacteria (Pelicic *et al.*, 1996a, b), problems have been reported with the high frequency of spontaneous sucrose resistance (Papavinasundaram *et al.*, 1998; Pavelka & Jacobs, 1999). A second screening marker is thus desirable.

The Kan^R colonies obtained were initially screened by plating them onto plates with 10 % sucrose and plates containing hygromycin, to distinguish between integration events (SCOs or DCOs) and spontaneous Kan^R arising after electroporation. Since the delivery vector we used is unable to replicate in mycobacteria, Hyg^R Suc^S clones were assumed to have integrated the plasmid into the chromosome by a SCO event. Analysis of the 150 transformants obtained showed that 51 (34 %) were straightforward DCOs and all the others were the result of SCOs. However, Southern blot analysis of eight of the DCO clones (Kan^R Hyg^S Suc^R) with Padh after *Bam*HI digestion did not confirm the expected genotype (Fig. 2a): hybridization resulted in two bands for all eight of the DCO candidate mutants. Although the ~ 4000 bp band that was consistent with a DCO event was present in each clone, we could also detect a ~ 3300 bp band that corresponded to the wild-type *adhC*. When *Bam*HI-digested DNA from these DCO clones was probed with

Pkan, only the ~ 4000 bp band was detected (Fig. 2b). On the other hand, when the blot was probed with PsacB no hybridization could be detected, confirming that the delivery vector had not been integrated into the chromosome of these clones, again suggesting a double recombination event (Fig. 2b).

The analysis of these results suggested that either the cassette from the delivery vector was integrated somewhere else in the chromosome, by illegitimate recombination or homologous recombination between two other sequences of the delivery vector, or the *adhC* gene was duplicated in the *M. smegmatis* genome. The illegitimate recombination possibility seemed very unlikely, as all the DCO clones had the same pattern when analysed by Southern blotting hybridization; however, we had no evidence that the analysed DCO events were actually homologous.

Analysis of some of the SCO clones showed that they resulted from a SCO event in the *adhC* coding region. Southern blot hybridization of eight SCO clones (Kan^R Hyg^R Suc^S) digested with *Bam*HI (Fig. 2c), *Apa*I and *Bgl*II (data not shown) and probed with Padh (Fig. 2c) revealed two types of SCO mutant, and it was possible to determine that they corresponded to SCO events that took place either upstream or downstream of the *adhC* gene (Fig. 2d). In the latter case an unexpected band was found to hybridize to Padh. It is possible that a wild-type *adhC* was also present in the clones in which the SCO event occurred upstream of *adhC*; however, since the band generated was the same size as the one generated by the *Bam*HI fragment containing the wild-type *adhC* (~ 3300 bp), only one band, corresponding to two DNA fragments, could be seen. These results argue against the existence of sequences other than *adhC* that are homologous to the plasmid in the *M. smegmatis* genome, and provide preliminary evidence that *adhC* could be duplicated within the *M. smegmatis* genome.

To confirm the duplication hypothesis, we repeated the electroporation of *M. smegmatis* competent cells with the delivery vector, using a two-step screening strategy. This enabled the selection of DCOs resulting from a second SCO event in a homologous SCO clone. If *adhC* was not an essential gene, a second cross-over event should have occurred in some of the SCO clones to generate a DCO clone in which *adhC* had been replaced by a disrupted copy of the gene. The presence of a non-disrupted copy of *adhC* together with a disrupted copy would confirm the hypothesis.

One of the SCO mutants selected on plates containing kanamycin and hygromycin, after having been characterized by Southern hybridization as a homologous SCO (data not shown), was picked and streaked out onto plates lacking antibiotics. Following cultivation, a loopful of cells was resuspended in liquid medium and serial dilutions were plated onto a sucrose-enriched medium to select for clones that had lost the integrated plasmid through a second SCO. Suc^R colonies were streaked onto kanamycin plates with and without

Fig. 2. Southern blot analysis of the *adhC* locus of representative clones resulting from homologous recombination with pAGA6KM. The clones were obtained by a one-step selection on agar plates containing sucrose and hygromycin. (a)

Fig. 3. PCR analysis of eight representative Kan^R Hyg^S Suc^R clones (nos 29–36) which resulted from homologous recombination with pAGA6KM. Altogether 100 clones were screened by PCR. The Kan^R Hyg^S Suc^R clones were obtained by a two-step selection on agar containing sucrose and hygromycin. PCR amplification was performed with primers R59 and G345. A 760 bp fragment was expected to be amplified from AdhC⁺ strains and a 1500 bp fragment from AdhC⁻ strains. Bands of 760 bp and 1500 bp were detected in each of the DCO mutants, suggesting the presence of two copies of the *adhC* gene within the *M. smegmatis* genome. As a control, PCR was performed on wild-type *M. smegmatis* (mc²155), resulting in the amplification of a 760 bp fragment.

hygromycin, and scored for growth to distinguish DCOs from SCO events which had acquired spontaneous resistance to sucrose. One hundred Kan^R Hyg^S Suc^R colonies were analysed by PCR (Fig. 3), but again both the 760 and

1500 bp bands were detected in each DCO mutant. Some of these clones were further analysed by Southern blotting, using the probes Padh and Pkan, which confirmed the results obtained by PCR (data not shown).

*Bam*HI-digested DNA from *M. smegmatis* wild-type (mc²155) and eight DCO clones (Kan^R Hyg^S Suc^R) hybridized to the Padh probe (*adhC* from pAGA5). (b) *Bam*HI-digested DNA from one DCO clone (Kan^R Hyg^S Suc^R) probed with both PsacB (*hsp60-sacB* from pAGA6KM) and Pkan (*aphA-3* from pUC18K). *Bam*HI-digested DNA from a SCO clone was also probed with PsacB and was used as a hybridization control. The *Bam*HI fragment from the SCO clone which contained *sacB* has 7400 bp. (c) *Bam*HI-digested DNA from *M. smegmatis* wild-type and eight Kan^R Hyg^S Suc^R clones probed with Padh. Clones 9, 10 and 16 correspond to SCO events that took place downstream of the *adhC* coding region; clones 11, 12, 13, 14 and 15 correspond to SCO events that took place upstream of the *adhC* coding region. (d) Schematic interpretation of the results shown in (c). Upper panel, a homologous SCO event upstream of the *adhC* coding region integrates the delivery vector into the chromosome in such a way that after *Bam*HI digestion, two fragments of 2829 bp and 3300 bp are obtained; Lower panel, when the homologous SCO event occurs downstream of the *adhC* coding region two fragments are obtained after *Bam*HI digestion (1508 bp and 4621 bp). The underlined *Bam*HI site does not exist in the genomic *M. smegmatis* DNA and was added during the construction of the delivery vector.

These results, together with the Southern blotting and PCR results for the first set of DCO mutants, strongly suggested that there were two copies of *adhC* in the *M. smegmatis* genome and that the DCO clones obtained with both screening strategies have the phenotype $\text{AdhC}^{+/-}$.

Southern hybridizations were carried out on genomic DNA from wild-type *M. smegmatis* which had been digested with several different restriction enzymes (among them *ApaI*, *BglII*, *EcoRV* and *SphI*). The blots repeatedly showed one single band for each of the enzymes when probed with *Padh* (results not shown). This suggested that the duplicated region, if any, should be bigger than the fragments generated when *M. smegmatis* DNA was digested with these different enzymes, i.e. 3–6 kb.

The definitive proof of the *adhC* duplication in the *M. smegmatis* genome was obtained when a clone in which two DCO events had occurred was isolated. Competent cells from clone 31, one of the first DCO mutants, were prepared and electroporated with the suicide delivery vector pAGA6KM. A two-step strategy was followed to screen DCO mutants, as the cells were already Kan^R and it was not possible to use kanamycin as the only selectable marker. SCO events were first selected on plates containing kanamycin and hygromycin; a second SCO was subsequently screened for by using the *sacB* and *hyg* genes. Five SCO mutants were picked and streaked out onto plates lacking antibiotics. Following growth, a loopful of cells was resuspended in liquid medium and serial dilutions were plated onto a sucrose-enriched medium. Seventy Suc^R colonies were streaked onto kanamycin plates with and without hygromycin to select for DCO mutants. Forty-nine $\text{Kan}^R \text{ Hyg}^S \text{ Suc}^R$ clones were selected and 27 were analysed by PCR. Among the tested clones only one (clone 18) showed the expected genotype for a double DCO event. The genomic DNA from this clone was extracted and analysed by Southern blotting (Fig. 4a). The probe *Padh* was used and was found to hybridize only to one *Bam*HI DNA fragment of ~4000 bp, which confirmed that the ~3300 bp band has been removed to become ~4000 bp, as expected from disruption of the second *adhC* gene. These results also demonstrated that *adhC* is not an essential gene for *M. smegmatis*.

Southern hybridizations of genomic DNA from *M. smegmatis* wild-type and from clone 31 digested with *Bam*HI were performed using the probe *Pdws*, a 1230 bp flanking region downstream of the *adhC* gene in *M. smegmatis* (Fig. 4b). This fragment is not present in the delivery vector pAGA6KM, but was initially cloned into pAGA5, and was used to verify whether the downstream flanking regions of the two *adhC* copies were the same. A band corresponding to ~3300 bp was obtained for the *M. smegmatis* wild-type strain, whereas two bands corresponding to ~3300 bp and ~4000 bp were obtained for the DCO clone. These findings indicated that *M. smegmatis* is merodiploid for *adhC* and probably for a certain number of neighbouring genes. It was

Fig. 4. Southern blot of *Bam*HI-digested DNA from clone 31 ($\text{AdhC}^{+/-}$), clone 18 ($\text{AdhC}^{-/-}$) and *M. smegmatis* mc²155. (a) *Bam*HI-digested DNA from wild-type *M. smegmatis* (mc²155), clone 31 and clone 18 hybridized to the *Padh* probe. *Padh* only hybridized to one ~4000 bp fragment for clone 18, indicating that the ~3300 bp *adhC* coding regions had been disrupted by insertion of the *aphA-3* cassette. (b) *Bam*HI-digested DNA from wild-type *M. smegmatis* (mc²155) and clone 31 probed with *Pdws*. *Pdws* was used to verify whether or not the downstream flanking regions of the two copies of *adhC* were the same. Clone 31 (DCO mutant) produced two bands (~3300 bp and ~4000 bp) which indicated that *M. smegmatis* is merodiploid for *adhC* and possibly for a certain number of neighbouring genes. (c) Schematic representation of the binding sites for the probes *Padh* and *Pdws*.

not possible to check the region upstream of *adhC* for merodiploidy, as the known upstream sequence of *adhC* was included in the delivery vector, pAGA6KM.

PFGE to determine the position of the two *adhC* genes

To determine the localization of the two *adhC* genes and obtain information about the size of this duplication in the *M. smegmatis* genome, agarose plugs containing DNA from *M. smegmatis* wild-type, clone 31 and clone 18, were digested with *DraI* and resolved by PFGE. By comparative Southern blot hybridization analysis, and thanks to the *DraI* restriction site in the kanamycin cassette used to disrupt the *adhC*, it was possible to obtain some information about the duplication within the *M. smegmatis* genome. Southern blot hybridization with P600, a 760 bp fragment of the ADHC ORF

Fig. 5. Southern blot analysis of a pulsed-field gel of *M. smegmatis* *mc*²¹⁵⁵, *M. smegmatis* *AdhC*^{+/−} (clone 31) and *M. smegmatis* *AdhC*^{−/−} (clone 18) genomic DNA digested with *Dra*I. (a) The Southern blot of wild-type *M. smegmatis* (*mc*²¹⁵⁵) and clone 31 *Dra*I-digested DNA was probed with P600, a 760 bp PCR fragment which hybridizes to the *adhC* ORF downstream of the site where the kanamycin cassette was inserted to disrupt the gene. Analysis showed that the size of each fragment containing the *adhC* copies within the *Dra*I fragment is at least ~250 kb, for the wild-type; for clone 31, two fragments were shown (~250 kb and ~20 kb) within the *Dra*I fragment. (b) The Southern blot of clone 18 *Dra*I-digested DNA was probed with P600 and with Pups. Probing with P600 generated a ~20 kb fragment; probing with Pups (a 343 bp fragment upstream of *adhC*) revealed a ~230 kb fragment within the *Dra*I fragment. (c) Schematic representation of what the data shown in (a) and (b) may possibly mean: a tandem duplication is thought to be the most likely explanation of these results. The relative position of each of the *adhC* genes, within the *Dra*I-digested DNA fragments, are shown.

downstream of the site where the kanamycin cassette containing the *Dra*I had been inserted, revealed that each *adhC* of *M. smegmatis* wild-type was within a ~250 kb *Dra*I DNA fragment. On the other hand, ~250 kb and ~20 kb fragments from clone 31, and a ~20 kb fragment from clone 18, were found to hybridize with P600 (Fig. 5a, b). The hybridization of the *Dra*I-digested DNA from clone 18 with Pups, a 343 bp fragment upstream of *adhC*, revealed a ~230 kb fragment (Fig. 5b). Though our results do not allow us to determine the exact size of this duplication, it may be at least ~250 kb. Fig. 5(c) shows our proposed interpretation of the results obtained by PFGE. We propose the existence of a tandem duplication as the most probable explanation; however, even if the duplication is not in tandem this does not affect the predicted size of the duplication found within the *M. smegmatis*

genome. The relative position of both of the copies of *adhC* in the *Dra*I fragments is shown in Fig. 5(c). The ~250 kb band obtained with P600 on *M. smegmatis* wild-type DNA, the ~20 kb band obtained with P600 on clone 18 DNA, and the ~230 kb band obtained with Pups on clone 18 should correspond to two *Dra*I DNA fragments each.

SDS-PAGE and Western blot analysis of the *M. smegmatis* *AdhC*^{+/−} and *AdhC*^{−/−} mutants

To verify whether the chromosomal mutations in *adhC* disrupted the synthesis of ADHC, lysates from 3 d pellicles of wild-type *M. smegmatis* and both recombinant strains were prepared, resolved by SDS-PAGE, and immunoblotted with 4B5, a murine monoclonal antibody raised against BCG-ADHC. As shown in Fig. 6,

Fig. 6. Detection of ADHC synthesis in extracts from *M. smegmatis* mc²155, *M. smegmatis* AdhC^{+/-} and *M. smegmatis* AdhC^{-/-} by Western blot analysis. Two micrograms of protein were separated on a 12% SDS-PAGE, electroblotted and detected by incubation with a murine monoclonal antibody (4B5) raised against the BCG-ADHC.

a single band of 38 kDa, corresponding to the ADHC, was found in extracts from *M. smegmatis* wild-type and from clone 31, the *M. smegmatis* AdhC^{+/-} mutant. However, this band was absent in lysates of the clone 18, the *M. smegmatis* AdhC^{-/-} mutant. The band corresponding to the *M. smegmatis* AdhC^{+/-} ADHC was fainter than the one from the wild-type strain, which indicates that in this mutant strain ADHC is expressed at a lower level than in the wild-type *M. smegmatis* strain. These results confirmed the results from the genetic experiments, suggesting the existence of two functional copies of *adhC* in *M. smegmatis* mc²155.

In vitro growth studies

The parent and mutant strains were cultivated in supplemented Middlebrook 7H9 broth (see Methods). The growth rate of both *M. smegmatis* AdhC^{+/-} and *M. smegmatis* AdhC^{-/-} were reduced when compared with the *M. smegmatis* wild-type, suggesting a growth disadvantage associated with the loss of expression of one or two *adhC* copies. On supplemented Middlebrook 7H10 broth, mutant strains produced after 2 d growth colonies with a different morphology when compared with the colonies produced by the wild-type strain (data not shown). However, the mutants were still acid-fast. Static cultures grown on Sauton medium from each of the mutants showed that pellicles from the knockout strains were less folded and more inclined to sink than the ones produced by the wild-type *M. smegmatis* (data not shown).

Fig. 7. Assay of the ADHC activity of *M. smegmatis* mc²155, AdhC^{+/-} and AdhC^{-/-}. Strains were grown as surface pellicles for 3 d, recovered by filtration, sonicated and centrifuged. The protein concentration within the cell lysates was determined using the method of Spector (1978) with BSA as a standard. ADHC activity was measured in 1 ml reaction mixtures containing 0.02 M KH₂PO₄/Na₂HPO₄ buffer (pH 7.3), 0.25 mM NADPH, 25 μg total protein extracted from *M. smegmatis* mc²155 (▲), *M. smegmatis* AdhC^{+/-} (■) or *M. smegmatis* AdhC^{-/-} (○), and (a) 200 μM benzaldehyde, (b) 100 μM octanal, (c) 1 mM butyraldehyde or (d) 200 μM cinnamaldehyde. ADHC activity was expressed as oxidation of 0.25 mM NADPH at A₃₄₀ (benzaldehyde, octanal and butyraldehyde) or at A₃₆₆ (cinnamaldehyde).

Biochemical characterization of the *M. smegmatis* AdhC^{+/−} and AdhC^{−/−} mutants

Lysates from 3 d pellicles of wild-type *M. smegmatis* and both recombinant strains were obtained, and ADH specific activity was determined by measuring the rate of oxidation of 0.25 mM NADPH at A_{340} in the presence of concentrations of octanal and benzaldehyde well above the K_m that had been previously determined for the Ms-ADHC *in vitro* (Galamba *et al.*, 2001). These two substrates had previously been shown to be, *in vitro*, the best available substrates for this enzyme. As can be seen in Fig. 7(a, b) the *M. smegmatis* AdhC^{−/−} could not reduce either of these two aldehydes, while *M. smegmatis* AdhC^{+/−} showed intermediate activities for both benzaldehyde and octanal, when compared with the *M. smegmatis* wild-type and *M. smegmatis* AdhC^{−/−}. We also tested *M. smegmatis* AdhC^{−/−} with two other aldehydes, butyraldehyde and cinnamaldehyde, but neither of them was reduced by the *M. smegmatis* AdhC^{−/−} lysates (Fig. 7c, d). The absence of activity in the double-knockout mutant could be explained either by the fact that ADHC is the only *M. smegmatis* ADH which uses NADPH as a cofactor, or that the other putative ADHs which can be deduced from the *M. smegmatis* genome sequence available (<http://www.tigr.org/tdb/mdb/mdbinprogress.html>) are either not expressed or are inactive in our crude bacterial extracts.

DISCUSSION

M. smegmatis ADHC mutants

The ability to inactivate genes by homologous recombination is a key requirement to study the function of individual genes. Although gene replacement in *M. smegmatis* is relatively efficient, some problems in getting gene mutations by standard gene replacement methods have been reported (Hinds *et al.*, 1999). Pretreatment of the DNA vector with UV light and the utilization of counter-selection markers have been described as useful methods to efficiently generate and screen mutants by allelic replacement. The former was reported to stimulate homologous recombination and abolish illegitimate recombination in recipient cells (Hinds *et al.*, 1999), and the latter was reported to enable the distinction of SCO, DCO and illegitimate recombination events (Parish & Stoker, 2000). In this study we used a UV-denatured suicide delivery vector containing the *adhC* gene disrupted by a kanamycin cassette, which prevents polar effects from the inserted antibiotic-resistance marker (Menard *et al.*, 1993). The delivery vector used to electroporate *M. smegmatis* mc²155 competent cells also contains two screening markers, *hyg* and *sacB*.

The initial attempts to select an ADHC knockout mutant were unsuccessful, though a large number of DCO mutants were generated. The one-step strategy initially used can be useful to generate marked mutations, but in some cases the required mutants will

not be obtained, e.g. if the gene is essential or the recombination frequency at the locus is low. In these cases, especially for slow-growing mycobacteria, where gene replacement has not proved to be straightforward (McFadden, 1996), a two-step strategy has been usually employed, whereby SCO events were first selected, and then screening for the second cross-over was carried out to yield the mutant strains. We thus decided to switch to a two-step strategy that should ensure that the DCO clones selected are homologous, and also help determine whether *adhC* is duplicated. DCO mutants were obtained from SCO clones where another recombination event had taken place. These DCO mutants were characterized and found to have the same genotype as the DCO mutants previously obtained with the one-step strategy. They were further characterized by Western blotting (data not shown) and seemed to have less ADHC present than the wild-type *M. smegmatis*. Altogether, our results provided strong evidence that there are two copies of the *adhC* in *M. smegmatis* mc²155.

This hypothesis was confirmed when competent cells of one DCO mutant were prepared and another DCO event was selected from this strain, using a two-step strategy. This led us to isolate one clone which, by both PCR and Southern blot hybridization analysis, was shown to have the two copies of *adhC* replaced by two disrupted copies. Western blot hybridization results confirmed the results obtained by Southern blot hybridizations. Thus, a defective mutant unable to express ADHC was only obtained when two functional native *adhC* copies were disrupted by homologous recombination.

These results showed that *adhC* is not an essential gene for the *in vitro* growth of *M. smegmatis*. Surprisingly, we observed a difference in the growth and morphology for both *M. smegmatis* AdhC^{+/−} and AdhC^{−/−} when compared with the wild-type AdhC^{+/+} strain. In the absence of further information about the physiological role of the ADHC enzyme these observations are difficult to explain.

The preliminary biochemical characterization of the *M. smegmatis* AdhC^{+/−} and AdhC^{−/−} mutants did not help us to find out which aldehydes are closest to the ones used *in vivo* by this mycobacterial ADH, as the AdhC^{−/−} knockout mutant does not use any of the structurally different substrates tested. Preliminary analysis of the lipid and aldehyde contents of the mutant strains compared to the wild-type *M. smegmatis* strain has not yet shown any significant differences.

M. smegmatis mc²155 genome duplication

Evidence accumulated during the generation of the *M. smegmatis* mc²155 *adhC* knockout mutants uncovered a large duplication in its genome. PFGE, which enables the separation of large DNAs and can be used to investigate genome organization, was used to obtain information about the duplication extension and

localization. Although PFGE is routinely used in many mycobacterial species, we were not successful in analysing *M. smegmatis* DNA by PFGE when using the classical protocols for mycobacteria (Levy-Frebault *et al.*, 1989; Philipp *et al.*, 1996). Similar difficulties had been previously described for the analysis of *Streptomyces lividans* DNA (Ray *et al.*, 1992), and were associated with Tris-dependent site-specific cleavage of the DNA. Tris-derived nucleolytic species have been shown to react with thiourea, which could thus protect the DNA from strand cleavage (Evans & Dyson, 1993). We achieved non-degradative PFGE of the *M. smegmatis* DNA only when 50 μ M thiourea was added to the running buffer. Southern blot analysis of *Dra*I-digested genomic DNA from *M. smegmatis* mc²155, clone 31 and clone 18 resolved by PFGE demonstrated that *M. smegmatis* mc²155 harbours a large chromosomal duplication whose size may be at least $\sim$ 250 kb. Our results did not prove that the whole $\sim$ 250 kb region was duplicated; however, *Dra*I sites are relatively rare in mycobacteria and it seems quite unlikely that two *Dra*I sites would exist in the *M. smegmatis* genome at the same distance from the two *adhC* genes, even if they did not belong to a duplicated region. The chances of the existence of two identical (same size and sequence) fragments running together in the gel is much greater than the existence of two fragments of the same size, but with different sequences. Thus, the size of the duplication found in the *M. smegmatis* genome could be at least $\sim$ 250 kb.

Chromosomal duplication and resultant gene duplication are ubiquitous features of genome evolution and have been viewed as the predominant mechanisms for the evolution of new gene functions and adaptive responses (Lupski *et al.*, 1996). Chromosomal duplications provide a means for increasing gene dosage and for generating novel functions from potential gene fusion events at duplication end points, and represent a source of redundant DNA for divergence. Over half of the proteins present in the tubercle bacillus have arisen from ancient gene duplication and adaptation events (Cole *et al.*, 1998; Tekaiia *et al.*, 1999).

The generally accepted model for the formation of chromosomal duplication in bacteria is that, after chromosomal replication, misaligned repeated sequences (e.g. *rrn* operons, IS elements, transposons) (Lupski *et al.*, 1996) or short DNA homologies (Edlund & Normark, 1981) act as substrates for homologous recombination, leading to duplication or deletion of the specific region between the repeated sequences. The duplicated regions are usually flanked by repeated sequences in a directed orientation, and duplication formation is generally found to be highly dependent on the RecA function. Certain duplications have been observed which confer a growth advantage under specific selective conditions and play a role in the adaptation of micro-organisms as a method for gene amplification. When conditions arise which cannot be compensated for by an alteration in gene expression, either in the laboratory or in nature, selection may

favour an increase in the copy number of a gene or a group of genes. In general these duplications are rather large, up to one-third of the chromosome, but they are highly unstable as they are typically tandem duplications and are lost by homologous recombination when environmental conditions change, thus representing a readily reversible source of genomic variation (Anderson & Roth, 1977). Spontaneous tandem chromosomal duplications are common in populations of *E. coli* and *Salmonella typhimurium* (Haack & Roth, 1995).

The existence of two tandem chromosomal duplications of the genome of *M. bovis* BCG Pasteur, DU1 (29 668 bp) and DU2 (36 161 bp) (Brosch *et al.*, 2000) was recently reported and showed that these genomic regions of the BCG genome are still dynamic. This study concluded that BCG Pasteur is diploid for at least 58 genes and that at a certain point in its evolution contained duplicate copies of a further 60 genes which were lost when a deletion internal to DU2 arose.

The presence of a large (> 250 kb) *cis* duplication in the *M. smegmatis* genome is described here for the first time. An amplification has already been described in *M. smegmatis*, in the gene *pstB*, encoding the putative ATPase subunit of the phosphate-specific transporter system, in a fluoroquinolone-resistant *M. smegmatis* strain (Bhatt *et al.*, 2000). However, this is one of those cases where selection for increased gene dosage resulted in spontaneous amplification of one gene and there was no evidence that other flanking genes were duplicated or amplified.

We cannot exclude the possibility that the duplication we describe here had originated as the result of specific environmental conditions, but this is certainly a large chromosomal duplication that includes many genes. We propose that the duplication we found is a tandem duplication because non-tandem or inverted duplications appear to be very rare compared with the tandem ones. In fact, most of the duplications that have been analysed in detail in bacteria appear to be tandem duplications (Anderson & Roth, 1977, 1981; Brosch *et al.*, 2000; Heath, 1992). The restriction analysis of *M. smegmatis* DNA with several restriction enzymes showed that this *M. smegmatis* chromosomal duplication is probably a recent one in which divergence of gene sequences has not yet occurred. The mechanism responsible for this duplication is still obscure. Since the assembled complete genome sequence of *M. smegmatis* is not yet available, we do not know which sequences could have been involved in the recombination event that originally generated this duplication.

A preliminary study of the sequencing and assembly of the complete *M. smegmatis* genome indicates that this, at nearly 7 Mbp, is approximately 50% larger than that of its slow-growing relatives (Merkel *et al.*, 2001). Since the genome sequence of *M. smegmatis* was based upon sequencing shotgun clones which do not detect possible genomic duplications or other genomic rearrangements, the previewed size for the genome of this mycobacterial species might have been underestimated. Bacterial arti-

ficial chromosome (BAC) libraries, representing the complete genome of *M. smegmatis*, could be used for detection and localization of other possible duplications. Quantitative DNA–DNA hybridization has also been reported as a method which allows accurate determination of the size of merodiploid chromosomes and the identification of genes present in two copies per chromosome (Hauser & Karamata, 1994).

Future strategies for disruption of *M. smegmatis* genes should consider the possibility of having to mutate more than one gene copy. Availability of the assembled complete genome sequence from *M. smegmatis* will enable the determination of which genes are included within the ~ 250 kb duplicated region, but additional techniques (such as BACs or heteroduplex mapping) will be needed to determine its exact size, orientation and origin, and to detect other duplications and other possible complex genomic rearrangements within the *M. smegmatis* genome.

ACKNOWLEDGEMENTS

We are grateful to Tanya Parish and Neil Stoker for generously supplying pGOAL19 and helpful suggestions, and to Robert Menard for providing pUC18K. We are also grateful to Ariane Deplano, Sabrina Damée and Eddie Van Bossuyt for their helpful advice with the PFGE experiments. This work was supported by grant BD/9181/96 from Fundação para a Ciência e Tecnologia, Portugal.

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Received 15 June 2001; revised 30 July 2001; accepted 14 August 2001.