Production of avirulent mutants of *Mycobacterium bovis* with vaccine properties by the use of illegitimate recombination and screening of stationary-phase cultures

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A better tuberculosis vaccine is urgently required to control the continuing epidemic. Molecular techniques are now available to produce a better live vaccine than BCG by producing avirulent strains of the *Mycobacterium tuberculosis* complex with known gene deletions. In this study, 1000 illegitimate recombinants of *Mycobacterium bovis* were produced by illegitimate recombination with fragments of mycobacterial DNA containing a kanamycin resistance gene. Eight recombinant strains were selected on the basis of their inability to grow when stationary-phase cultures were inoculated into minimal medium. Five of these recombinants were found to be avirulent when inoculated into guinea pigs. Two of the avirulent recombinants produced vaccine efficacy comparable to BCG against an aerosol challenge in guinea pigs with *M. bovis*. One of these recombinants had an inactivated *glnA2* gene encoding a putative glutamine synthetase. Transcriptional analysis showed that inactivation of *glnA2* did not affect expression of the downstream *glnE* gene. The other recombinant had a block of 12 genes deleted, including the sigma factor gene *sigG*. Two avirulent recombinants with an inactivated *pckA* gene, encoding phosphoenolpyruvate carboxykinase which catalyses the first step of gluconeogenesis, induced poor protection against tuberculosis. It is clear that live avirulent strains of the *M. tuberculosis* complex vary widely in their ability as vaccines to protect against tuberculosis. Improved models may be required to more clearly determine the difference in protective effect between BCG and potential new tuberculosis vaccines.

Keywords: tuberculosis, *Mycobacterium tuberculosis*, vaccines, virulence, BCG, guinea pig

INTRODUCTION

Tuberculosis exacts enormous suffering and death on a global basis. Most disease is caused by *Mycobacterium tuberculosis*, but in many parts of the world a significant amount of disease is caused by the very closely related organisms *Mycobacterium africanum* and the major animal pathogen *Mycobacterium bovis*. Together with a few less important species, these organisms are collectively referred to as the *M. tuberculosis* complex.

Many factors contribute to this global burden of tuberculosis, but one cause is undoubtedly the lack of a more effective vaccine. The current vaccine, Bacille Calmette–Guérin (BCG), is an attenuated strain of *Mycobacterium bovis* that was developed 80 years ago by the simple technique of subjecting a virulent *M. bovis* strain to serial culture *in vitro* for 13 years (Brewer & Colditz, 1995). BCG has become accepted to the extent that there have been at least 3 billion doses administered, more than any other vaccine (Bloom & Fine, 1994). While BCG undoubtedly prevents childhood forms of the disease, it provides only limited protection against the vast majority of disease and in the most recent large

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Abbreviation: PPD, purified protein derivative.

trial it provided no protection at all (Bloom & Fine, 1994; Brewer & Colditz, 1995). Although it has been difficult to demonstrate that BCG has had any major effect on reducing the incidence of disease (Fine, 1995), the very wide and continuing use of BCG and its ability to limit the severity of disease in animal models has made it the gold standard against which new experimental vaccines are compared.

In the last 10 years, an increased concern about tuberculosis together with many developments in immunology and molecular biology has aroused intense interest in producing better vaccines against the disease. There is general agreement that a successful vaccine will need to induce a type 1 immune response, but the detailed combination of responses that are required to induce optimum levels of protective immunity is not known (Snewin *et al.*, 2000). This has made it difficult to specify which features of a vaccine are most important and therefore which particular type of vaccine is likely to prove most effective. A large range of possible types of both living and non-living vaccines are being actively researched (Collins, 2000; Snewin *et al.*, 2000) and different types of vaccines may be useful for different purposes.

One approach is to modify the current vaccine and already a genetically modified form of BCG has been reported to produce better protection than BCG (Horwitz *et al.*, 2000). The genetic causes of attenuation in BCG remained completely unknown until 6 years ago when it was shown that a block of nine genes, including the *esat6* gene, was missing in BCG but was present in other strains of the *M. tuberculosis* complex (Mahairas *et al.*, 1996). More recently, specific inactivation of the *esat6* locus was shown to cause partial, but not complete attenuation of an *M. bovis* strain (Wards *et al.*, 2000), and other blocks of genes that are absent (Behr *et al.*, 1999; Gordon *et al.*, 1999) or duplicated (Brosch *et al.*, 2002) in BCG have been identified. Add to this the likely presence of mutated genes which are yet to be identified and it is evident that considerable further work will be required to elucidate the role of the many genetic differences between BCG and clinical tuberculosis strains.

An alternative method of making a new live tuberculosis vaccine is to attenuate a virulent strain of the *M. tuberculosis* complex by introducing one or more easily identifiable mutations and several groups have shown that vaccine candidates can be produced in this way (de Lisle *et al.*, 1999; Jackson *et al.*, 1999; Hondalus *et al.*, 2000). In the early stages of carrying out allelic exchange of *ahpC* in an *M. bovis* strain (Wilson *et al.*, 1997), using an approach that was eventually successful (Collins, 2001), we had produced moderate numbers of illegitimate recombinants and investigated their potential use as tuberculosis vaccines. A total of 440 illegitimate recombinants were screened on minimal medium and four mutants were selected, all of which were found to be attenuated in guinea pigs. When guinea pigs inoculated with these mutants were challenged with virulent

M. bovis, two of the mutants induced a level of protection that was in some respects better than that induced by BCG, although the differences were not significant (de Lisle *et al.*, 1999). In one of these mutants, a putative undecaprenol kinase gene was interrupted by illegitimate insertion of a DNA fragment that accompanied a 2 bp chromosomal deletion and, in the other, DNA fragment insertion was accompanied by a large DNA deletion of 15 kb containing 12 genes. None of the four mutants appeared to have any interrupted genes encoding enzymes for common metabolic pathways that are often associated with auxotrophy, such as amino acid, purine, pyrimidine or co-factor synthesis. Subsequently, we found these mutants were not true auxotrophs as 2-week-old cultures in complete medium were able to grow when inoculated into minimal medium. Re-examination of procedures revealed the mutants in the study had been subcultured into minimal medium from 5- to 10-week-old cultures in complete medium, at a stage when they would have been in stationary growth. Since the ability of strains of the *M. tuberculosis* complex to survive in the host in a stationary or lowered metabolic state is regarded as a crucial facet of tuberculosis pathogenesis (Colston & Cox, 1999), we reasoned that it might be desirable to have a vaccine strain without this ability.

Regardless of this explanation, the use of 5- to 10-week-old cultures had produced two mutants with vaccine effectiveness at least equal to that of BCG, so a similar approach was used to produce mutants in this study. Eight recombinants were obtained by screening 1000 illegitimate mutants of *M. bovis* and these were produced by using either the original *ahpC* fragment or a second fragment made from another *M. bovis* gene. Identification of the mutated regions and virulence and vaccine testing of the strains was performed and two strains with vaccine properties at least as good as those of BCG were obtained.

METHODS

***M. bovis* strains and culture conditions.** *M. bovis* ATCC 35723, the strain used for illegitimate recombination, and *M. bovis* WAg201, the strain used to challenge animals in vaccine studies, were cultured in Middlebrook 7H9 (Difco) liquid medium supplemented with albumin/glucose complex (ADC; Difco), 0.05% Tween 80 and 0.4% sodium pyruvate, and on Middlebrook 7H11 (Difco) solid medium supplemented with ADC and 0.4% sodium pyruvate. Recombinants were isolated on 7H11 medium supplemented with 2.5% bovine serum and 20 µg kanamycin ml⁻¹. For screening of recombinants in minimal medium, 5 µl of a 5- to 8-week-old culture in supplemented Middlebrook 7H9 medium was inoculated into both supplemented Middlebrook 7H9 medium and into Proskauer & Beck minimal liquid medium (Darzins, 1958) supplemented with 0.05% Tween 80 and 0.2% sodium pyruvate. Recombinants that did not grow in minimal medium were rescreened twice to confirm their stability. For culture of *M. bovis* from guinea pig spleens, half the spleen was homogenized with 20 ml water for 1 min. This was filtered through sterile cheese cloth and centrifuged at 3500 g for 20 min. The pellet was resuspended in 0.5 ml water and

Fig. 1. Constructs used for illegitimate recombination. (a) DNA fragment containing *ahpC/ahpD* and *neo* (kanamycin resistance); (b) DNA fragment containing *Rv3844/Rv3845* genes and *neo*.

aliquots were plated onto Middlebrook 7H11 supplemented with 0.6 ml oleic acid l⁻¹, 50 g bovine serum albumin l⁻¹, 20 g glucose l⁻¹, 7.7 g NaCl l⁻¹, 0.4% sodium pyruvate, 0.5% lysed sheep red blood cells, 10% bovine serum, 10 µg fungizone ml⁻¹, 200 IU polymixin B sulphate, 100 µg tartracillin ml⁻¹ and 10 µg trimethoprim ml⁻¹.

Techniques for making recombinants and determining mutation sites. The DNA fragment containing the interrupted *ahpC* gene (Fig. 1a) used for illegitimate recombination was prepared by *NotI* digestion as described previously (Wilson *et al.*, 1997). A DNA fragment containing the *Rv3844* gene was also prepared. Briefly, a 2593 bp region of *M. bovis* (homologous to *M. tuberculosis* at position 13157–15749 of Mtcy1A6; GenBank accession no. Z83864) was amplified using PCR primers containing *PacI* sites and cloned into pUHA9, a derivative of pBluescript KSII+ (Stratagene) which contains a *PacI* cloning site. A kanamycin resistance gene (*neo*) was inserted at the *AatII* site of *Rv3844* by appropriate DNA digestion, blunt-end ligation and cloning. For electroporation, the plasmid containing this construct was digested with *StuI* and *NotI* which cut at positions equivalent to 13257 and 15209 respectively in the 13157–15749 fragment of Mtcy1A6 to produce a 3.4 kb fragment (Fig. 1b). The *ahpC* and *Rv3844* plasmid digests were precipitated and washed in ethanol to remove electrolytes and electroporated into *M. bovis* using a high efficiency technique at 37 °C as described

previously (Wards & Collins, 1996). The electroporated cells were cultured on solid medium containing kanamycin and individual colonies were subcultured in liquid medium containing 20 µg kanamycin ml⁻¹.

DNA was extracted (van Soolingen *et al.*, 1991) from liquid cultures of strains identified by their failure to grow on minimal medium, digested with *EcoRI*, separated on a 20 cm 1% agarose gel and transferred to nylon (Zeta probe; Bio-Rad) by Southern blotting. Nylon filters were hybridized with probes made by incorporating [³²P]dCTP into a plasmid containing the *neo* gene by nick translation (Life Technologies). To determine the site of insertion of the DNA fragment in the chromosome of a recombinant, chromosomal DNA was digested with a restriction enzyme that did not cut within the inserted fragment (*XhoI* for the *Rv3844* fragment and *EcoRI* or *BamHI* for the *AhpC* fragment), ligated into the appropriate site of pBluescript KSII+, electroporated into *Escherichia coli* XL-1 Blue MR and plated on Luria–Bertani medium containing 25 µg kanamycin ml⁻¹ and 50 µg ampicillin ml⁻¹. The junction regions of the fragment and chromosome were sequenced, using appropriate primers directed outwards from the vector sequence into the mycobacterial sequence. DNA sequences were analysed using the programs of the Genetics Computer Group (Madison, WI, USA) and compared to GenBank (www.ncbi.nlm.nih.gov) and Sanger (www.sanger.ac.uk) databases.

Transcription of *glnA2*. Exponential-phase cultures of *M. bovis* ATCC 35723 and WAg530 were harvested by centrifugation and RNA was extracted by cell disruption of 10⁸ c.f.u. in the presence of 1 ml Trizol (Invitrogen) and 0.5 ml zirconium beads. After Trizol extraction, residual DNA was removed by incubation in DNaseI (Invitrogen) and RNA was further purified using an RNeasy Mini Kit (Qiagen). Reverse transcription was performed using gene-specific primers (designated RT-PCR in Table 1) and *C. therm* polymerase (Roche), according to the manufacturer's instructions. PCR was performed in either a GeneAmp PCR System 9600 (Applied Biosystems) or a GeneAmp PCR System 9700 (Applied Biosystems) in 9600 emulation mode using the following programme: 1 cycle of 94 °C for 5 min; 32 cycles of 94 °C for 45 s, 60 °C for 30 s, 72 °C for 60 s; and 1 cycle of 72 °C for 7 min.

Determination of virulence. All animal work was approved by the institution's Animal Ethics Committee. The virulence of each *M. bovis* strain was tested in three female Duncan–Hartley guinea pigs, as described previously (Wilson *et al.*,

Table 1. Primers used for RT-PCR of the *glnA2* locus

Gene	Primer	Use	Nucleotide sequence
<i>glnE</i>	DMC419	RT-PCR*	GGGCATACGACATCCAGTCC
<i>glnE</i>	DMC420	PCR	TGGGCGAAGGCGGCTACATC
<i>glnA2-glnE</i>	DMC421	RT-PCR	CTCGTCCCATCCGGTGTCTG
<i>glnA2-glnE</i>	DMC422	PCR	AGGCCGAGGACAACGTATGG
<i>glnA2</i> post-mutation	DMC527	RT-PCR	GGTAGTTCGCCCCTCCGTG
<i>glnA2</i> post-mutation	DMC451	PCR	TACACGCCGCACAAGACCTC
<i>glnA2</i> pre-mutation	DMC452	RT-PCR	ACCCACTTCCGACAGCTGCA
<i>glnA2</i> pre-mutation	DMC453	PCR	GCTACGTGCATCCCGAAATC

* Primer used for reverse transcription as well as PCR.

1995). Animals were inoculated by injecting 10^6 c.f.u. subcutaneously into their flank and were sacrificed at 8 weeks. The assessment of virulence was based on the presence of visible lesions of tuberculosis, particularly in the spleen. All guinea pigs were skin-tested by the intradermal injection of 4 units (0.1 ml) of bovine purified protein derivative (PPD; Agriquality) immediately prior to inoculation with *M. bovis* and immediately prior to the animals being euthanased.

Determination of vaccine efficacy. For vaccination studies, groups of six Duncan–Hartley guinea pigs were vaccinated by subcutaneous injection in the right flank with 10^5 c.f.u. of one of the avirulent recombinant strains. A group of 12 animals was vaccinated with BCG and a control group of 12 animals was not vaccinated. Eight weeks post-vaccination, all animals were challenged with a single cell suspension of *M. bovis* WAg201 prepared by sonication for 30 s and filtration through an 8 µm membrane filter. Guinea pigs were infected via the respiratory route by using an aerosol chamber which produces droplet nuclei of the size appropriate for entry into alveolar spaces (McMurray *et al.*, 1985; Wiegeshaus *et al.*, 1970). The concentration of viable *M. bovis* in the nebulizer fluid was empirically adjusted to result in the inhalation and retention of 2–10 viable organisms per guinea pig. The aerosolized solution contained approximately 4.8×10^4 c.f.u. ml⁻¹. The requirement for this challenge dose had previously been estimated from the number of primary tubercles observed grossly in the lungs of non-vaccinated guinea pigs at 4 weeks post-infection. Five weeks after challenge, the animals were euthanased and autopsied, and body weight and gross pathology were recorded. Samples of spleen were subjected to mycobacterial culture and enumeration. Delayed type hypersensitivity to tuberculin injected intradermally was measured immediately before vaccination and prior to the animals being sacrificed using bovine PPD. Statistical analyses by ANOVA were performed on spleen weights and on log₁₀ transformations of spleen and lung bacterial counts and numbers of macroscopic lesions in the spleen.

RESULTS AND DISCUSSION

Isolation of recombinants and determination of mutation sites

Five electroporations into *M. bovis* were performed for each fragment and a total of 1000 recombinants were recovered, 325 from the *ahpC* fragment electroporations and 675 from the *Rv3844* fragment electroporations. Strains that were unable to grow in minimal medium, but did grow in the supplemented Middlebrook 7H9 medium were rescreened twice and eight recombinants were obtained. A Southern blot hybridization of DNA from these eight strains and one recombinant that failed the selection is shown in Fig. 2. Subsequent testing of actively growing cultures of these eight strains showed that none of them was an auxotroph, although WAg530 and WAg531 did grow more slowly in minimal medium than the other recombinants. While none of the recombinants had identical insertions, analysis showed that in some cases insertion into the same region of the chromosome had occurred.

The fragment insertion site in the *M. bovis* chromosome of each of the eight recombinants was determined after cloning a region of the chromosome which contained

Fig. 2. Southern blot hybridization after *Eco*RI digestion and probing with *neo* of eight recombinants that were unable to grow in minimal medium (lanes 1–5, 7–9) and one recombinant that failed the screen and grew in minimal medium (lane 6).

the inserted fragment and flanking sequences. The initial sequencing primers used were located near each end of the fragment with their 3' ends directed outwards so that the DNA which flanked the inserted fragment would be sequenced. In some of the recombinants there had been a deletion of the inserted fragment such that one of the initial primers did not bind. Other primers were subsequently used that were complementary to sequences further into the fragment until DNA sequence flanking both sides of the fragment was obtained. The insertion sites of the eight recombinants are given in Table 2 relative to the *M. tuberculosis* genome sequence in the Sanger database. The size of the DNA that was deleted from the chromosome during the insertion and the mean number of spleen lesions per guinea pig are also given. The chromosomal regions in which the fragments inserted and an illustration of the genes affected are shown in Fig. 3. The disruption ranged from one gene in WAg527 to 12 genes in WAg526. Three recombinants (WAg527, WAg528, WAg531) had insertions in the same region of the chromosome with deletions ranging from zero to almost 11 kb (Fig. 3a), two recombinants (WAg526 and WAg529) had insertions in a different region of the chromosome (Fig. 3b) and the remaining three recombinants had insertions in widely separated parts of the chromosome (Fig. 3c–e). None of the insertions was in the same regions as those of the four recombinants produced in our earlier work (Wilson *et al.*, 1997) and none of the deletions overlaps any of the blocks of genes that have been shown to be present in some strains of the *M. tuberculosis* complex, but absent in *M. bovis* or other strains of the complex (Behr *et al.*, 1999; Gordon *et al.*, 1999). The region in which the fragments had inserted did not appear to depend on the fragment used, as recombinants in the Rv0209–Rv0216 region (Fig. 3a) were produced by both fragments. As in our earlier work, the amount of gene

Table 2. Details of the eight selected recombinants and the parent strain

Strain (WAg)	Fragment used	Mean no. of spleen lesions per animal	Deletion size (bp)	No. of genes interrupted	Deletion region
525	<i>Rv3844</i>	34	214	1	34004–34217 of <i>Mty13E12</i>
526	<i>Rv3844</i>	0	11 227	12	16862–28088 of <i>MtcI28</i>
529	<i>Rv3844</i>	2	7 123	8	19999–27121 of <i>MtcI28</i>
527	<i>ahpC</i>	0	0	1	Insert at 5424 in <i>Mtcy8D5</i>
528	<i>Rv3844</i>	0	10 860	9	2270–13129 of <i>Mtcy8D5</i>
531	<i>ahpC</i>	0	2 909	2	4354–7262 of <i>Mtcy8D5</i>
530	<i>ahpC</i>	0	30	1	5330–5359 of <i>Mtcy427</i>
532	<i>ahpC</i>	38	3 989	2	4628–8616 of <i>Mtv035</i>
Parent	–	65	–	–	–

Fig. 3. Sites of illegitimate recombination in *M. bovis* of the eight selected recombinants. (a) WAg527, WAg528 and WAg531; (b) WAg526 and WAg529; (c) WAg525; (d) WAg530; (e) WAg532. Deletion and insertion are shown relative to the annotation of *M. tuberculosis* H37Rv.

deletion varied widely from 11 kb to no deletion (WAg527) and in some cases there had also been large deletions from the end of the inserting fragment. In the case of WAg527, parts of the *NotI* sites delineating the inserting fragment were still present and the insertion was accompanied by a chromosomal duplication of 9 bp so that each end of the insertion was flanked by the same 9 bp sequence. Although the inserting fragment has no homology to transposases, this duplication is very

reminiscent of the 8 bp duplications without deletion that occur on transposition of Tn5367 into the chromosome of the *M. tuberculosis* complex (Bardarov *et al.*, 1997). Interestingly, the duplicated 9 bp sequence in WAg527 contains a GGCGCC palindrome which has been shown to be a highly preferred recombination site for illegitimate recombination in *Rhodococcus fascians* (Desomer *et al.*, 1991), although in that study no duplication at the sites of recombination occurred. None

Table 3. Vaccination results

a, b, c, d, Values within the same column with the same suffix are not significantly different; ND, not determined.

<i>M. bovis</i> recombinant	Mean spleen weight $\pm$ SE (g)	Mean no. of spleen lesions $\pm$ SE	log(Spleen <i>M. bovis</i> c.f.u.) $\pm$ SE	log(Lung <i>M. bovis</i> c.f.u.) $\pm$ SE
Non-vaccinated	2.50 $\pm$ 0.33 ^a	46 $\pm$ 10 ^a	4.97 $\pm$ 0.17 ^a	4.22 $\pm$ 0.25 ^a
BCG	1.29 $\pm$ 0.09 ^c	0.25 $\pm$ 0.18 ^d	1.53 $\pm$ 0.53 ^c	2.36 $\pm$ 0.36 ^b
WAg526	1.17 $\pm$ 0.10 ^c	0.0 $\pm$ 0.0 ^d	1.21 $\pm$ 0.21 ^c	2.41 $\pm$ 0.47 ^b
WAg527	1.82 $\pm$ 0.16 ^{bc}	26 $\pm$ 17 ^{bc}	3.46 $\pm$ 0.37 ^b	4.19 $\pm$ 0.25 ^a
WAg528	1.97 $\pm$ 0.27 ^{ab}	36 $\pm$ 12 ^{ab}	3.40 $\pm$ 0.69 ^b	3.83 $\pm$ 0.16 ^a
WAg531	1.35 $\pm$ 0.17 ^{bc}	1.7 $\pm$ 1.3 ^{cd}	ND	ND
WAg530	1.15 $\pm$ 0.10 ^c	0.0 $\pm$ 0.0 ^d	ND	ND

of the other seven *M. bovis* recombinants had this palindrome at or within 9 bp of their insertion sites.

Analysis of mutations in relation to virulence

The mean number of spleen lesions per guinea pig was used to indicate the virulence of the recombinants (Table 2). Five of the recombinants caused no spleen lesions and were used for vaccination studies. One of the recombinants had greatly reduced virulence and the remaining two recombinants had a level of virulence about half that of the parent strain (Table 2). Lesions in the liver were also recorded but were less sensitive for assessing virulence. None of the five recombinants in Table 3 produced any liver lesions, the parent strains produced a mean of three to five liver lesions per animal and WAg525 and WAg532 produced liver lesions in one guinea pig each. No delayed type hypersensitivity reactions were observed when the animals were skin-tested with PPD prior to being inoculated with the *M. bovis* recombinants. All animals had positive skin test reactions immediately prior to sacrifice, indicating that they had been successfully infected.

WAg527, WAg528 and WAg531 were all avirulent and all have an inactivated *pckA* gene. In the case of WAg527, this is the only gene inactivated and the gene annotation of this region (Fig. 3a) indicates that polar effects on nearby genes are unlikely. The avirulence of WAg528 and WAg531 could also be due entirely to the inactivation of *pckA*, but the other known and unknown genes that are also inactivated in these two recombinants may also affect their virulence. *pckA* encodes phosphoenolpyruvate carboxykinase that catalyses the decarboxylation and phosphorylation of oxaloacetate to form phosphoenolpyruvate. This is the first committed step of gluconeogenesis in bacteria and the enzyme is utilized during gluconeogenic growth when sugar levels are low (Riedel *et al.*, 2001; Matte *et al.*, 1997). The inability of WAg527 to cause any disease indicates that the gluconeogenic pathway is essential for the virulence of *M. bovis* in guinea pigs.

WAg526 and WAg529 both have large deletions at the

sigG locus (Fig. 3b). WAg529 had greatly reduced virulence and WAg526 was avirulent. Since the deletion in WAg529 is nested within that of WAg526, it can be concluded that one or more of the extra genes of unknown function that were deleted in WAg526 (*Rv0175*–*Rv0178*) contribute to loss of virulence as well as at least one of the genes absent in both recombinants. Of the two genes of known function identified as inactivated in both recombinants, *sigG* is a sigma factor gene belonging to the family which regulates extracytoplasmic functions and stress responses and *bglS* encodes a β -glucosidase. Neither of these genes has been studied in detail but it has been reported that the expression of *sigG* is strongly down-regulated under conditions of low aeration (Manganelli *et al.*, 1999). The 5- to 10-week-old cultures used for screening would have had lowered levels of oxygen and the inability of these two strains to grow when transferred to fresh minimal medium as well as their considerable loss of virulence may indicate that *sigG* plays a significant role when the bacteria are returning from lower to higher levels of oxygen, a situation that may also occur *in vivo*.

WAg530 has a small deletion in the *glnA2* gene encoding a putative glutamine synthetase and was found to be avirulent. Two other genes in the same chromosomal region (Fig. 3d), *glnA2* and *glnE*, are also involved in glutamine synthesis. The glutamine synthetase encoded by *glnA1*, which has been extensively studied, is not actively exported from *M. tuberculosis*, but is found in the extracellular medium of *M. tuberculosis* cultures and may play a role in pathogenesis (Tullius *et al.*, 2001). The product of *glnE* is a regulator that by comparison to homologous enzymes in *Streptomyces coelicolor* controls *glnA1*, but not *glnA2* (Parish & Stoker, 2000). *glnE* has been shown to be essential (Parish *et al.*, 2001), but *glnA2* has not been studied. In the annotation of DNA sequences from *M. tuberculosis*, *glnA2* reads in the same direction as *glnE* and its coding sequence ends only 48 bp before the initiation codon of *glnE*. This region is identical in the *M. bovis* genome. This raised the possibility that these two genes may be in an operon and may be expressed co-ordinately. To test this possibility, we measured RNA production at various points in this

Fig. 4. Transcriptional characterization of *glnA2* and *glnE* in *M. bovis* WAg530 and its parent strain ATCC 35723. (a) Alignment of the four primer pairs used for RT-PCR; large arrowheads indicate the primers used for reverse transcription. (b) Ethidium-stained agarose gel of PCR products from the four RT-PCR reactions. Products of the expected size were obtained in all cases. There was no evidence of any product for primer pair 3 in WAg530. Control reactions (not shown) utilizing DNaseI-treated RNA without reverse transcription failed to yield PCR products, while all primer pairs gave the expected PCR product when used to amplify genomic DNA.

region of the chromosome (Fig. 4). RNA transcription of *glnA2* upstream of the insertion site and of *glnE* occurred in both WAg530 and its parent strain and there was no transcript running from *glnA2* to *glnE* in either strain. The only transcription difference between the strains was the absence in WAg530 of any *glnA2* transcription downstream of the illegitimate insertion site in *glnA2*. These results indicate that *glnA2* and *glnE* are not in an operon and are consistent with the earlier finding that *glnE* is an essential gene (Parish *et al.*, 2001). The loss of virulence in WAg530 is thus likely to be entirely due to disruption of *glnA2* and not to downstream polar effects on *glnE*.

One of the virulent recombinants, WAg525 (Fig. 3c), had a deletion in a small gene of unknown function and was not further analysed. In the other recombinant that retained virulence, WAg532 (Fig. 3e), two PE-PGRS genes had been largely deleted (Fig. 3e). Cole *et al.* (1998) found that these genes are part of a big subfamily of 61 genes that together with the other 38 members of the PE family and the unrelated 68 members of the PPE family make up about 10% of the genome. Their proposal that these families may play a role in influencing the host's immune responses has received some experimental support (Delogu & Brennan, 2001) and the putative proteins of the two genes inactivated in WAg523 are highly similar at their N-terminal ends to a PE-PGRS protein that binds fibronectin and is recognized immunologically by tuberculosis patients (Espitia *et al.*,

1999). There is also evidence that inactivation of some of these genes can affect infection of macrophages (Brennan *et al.*, 2001) and this also occurs when similar PGRS genes are inactivated in the related pathogen *Mycobacterium marinum* (Ramakrishnan *et al.*, 2000). The genes inactivated in WAg523 clearly had a phenotypic effect expressed under the screening regime but this had little apparent effect on virulence of WAg523 in guinea pigs. The guinea pig model gives a measure of acute disease and one possible explanation is that, given the way the strain was selected, the mutation has affected the ability of the strain to cause reactivation tuberculosis, a property that could only be tested in a reactivation animal model such as the Cornell model (Colston & Cox, 1999). A superficial argument against this explanation is that both of these genes are absent from *M. tuberculosis* CDC1551, a recent clinical isolate (Betts *et al.*, 2000). However, the complement of genes differs between strains of the *M. tuberculosis* complex and the differences appear greater between *M. tuberculosis* and *M. bovis* strains than between different *M. tuberculosis* strains (Behr *et al.*, 1999; Brosch *et al.*, 2002; Gordon *et al.*, 1999). This situation, together with the similarity between PE-PGRS genes, indicates a likelihood that the absence of particular PE-PGRS genes in one strain may be compensated by the presence of other genes.

Vaccination study

No delayed type hypersensitivity reactions were observed when the animals were tested prior to vaccination. All animals had positive skin test reactions immediately prior to sacrifice and there were no statistical differences between the responses of the groups of challenged animals, including the non-vaccinated group. Macroscopic lesions consistent with tuberculosis were observed in the lungs of the guinea pigs challenged with virulent *M. bovis* and the protection afforded by the vaccines against spread of infection was judged by the size of the spleens, the number of spleen lesions and the log(transformed c.f.u. of *M. bovis*) in the spleen and lung (Table 3). Cultures were not available for WAg530 and WAg531. There were a few liver lesions in most of the unvaccinated control animals, and in some animals vaccinated with WAg528, but they were not sufficient to make these groups significantly different from the other vaccinated groups that had no liver lesions. The five recombinants that were tested gave widely varying levels of protection against the spread of infection. WAg527 and WAg528 provided relatively poor protection, although the spleen c.f.u. were significantly different from controls. WAg531 gave intermediate protection. The best protection was provided by BCG, WAg526 and WAg530 and these could not be distinguished statistically ($P < 0.05$). No spleen lesions were found for WAg526 and WAg530.

As in our earlier study (Wilson *et al.*, 1997), several recombinants have produced results that, while not significantly different from those for BCG, do have some indices of protection that indicate a lower spread of

infection than that achieved by BCG. In some recent vaccine models of tuberculosis in which animals were first exposed to environmental mycobacteria, BCG provided little or no protection against tuberculosis (Brandt *et al.*, 2002; Buddle *et al.*, 2002), although in the second of those studies chemical mutants of *M. bovis* gave significant protection. These differences in protection may result from differences in persistence between BCG and other avirulent strains or from stimulation of a qualitatively different immune response. Whether the strains developed in this study would give significantly better protection than BCG if one of these more recent vaccine models was used or if the vaccination protocol was altered in some other way to exemplify the differences between protective vaccines requires further experimentation. The two recombinants that gave poorer protection (WAg527 and WAg528) are both strains that lack an active *pckA* gene. WAg531, which also lacks an active *pckA* gene, provided protection that was not significantly different to that provided by BCG, although it did allow a higher mean spleen weight and lesion count than BCG, WAg526 and WAg530. These results show that while inactivating *pckA* does create avirulent strains of *M. bovis*, those strains do not appear to have the potential to provide a better vaccine than BCG. More generally, it also shows that avirulent strains of the *M. tuberculosis* complex are not all able to stimulate a good level of protection against tuberculosis even when their inoculation into guinea pigs results in normal skin test reactivity.

The results described here support the approach used in this and the previous study (Wilson *et al.*, 1997; de Lisle *et al.*, 1999) of screening stationary-phase cultures for their ability to grow in minimal medium and testing the virulence and vaccine potential of strains identified by this selection. Together with our earlier study, a total of 12 recombinants (0.8%) have been isolated from 1440 tested of which nine (0.6%) are avirulent. None of the genes inactivated in these nine avirulent strains has been reported in other studies as being important for virulence of strains of the *M. tuberculosis* complex. Several of the recombinants provide protection against the spread of tuberculosis that, while not significantly different to that provided by BCG, results in slightly better indices of protection than BCG. Whether the recombinants produced here would provide better protection against tuberculosis than BCG if they were tested under more stringent conditions remains to be determined, but the current results encourage belief that a better live vaccine than BCG may be attainable.

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