

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO	
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DEP: 2020 DIRECCION DE NUEVAS CREACIONES	EVE: 378 FASE NACIONAL INCLUIDO CAPITULO I
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II	FOLIOS: 17
ACT: 519 PRESENTACION	

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado(a)/Señor(a)
ALICIA LLOREDA RICAURTE

Asunto: Radicación: 11-9237- -7
Trámite: 11
Evento: 378
Actuación: 519
Oficio: 16704
Folios: 17

Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	X	Capítulo I	X	Capítulo II	
No. Solicitud Internacional	PCT/EP2009/059585				
Fecha de Presentación Internacional	24/07/2009				

Como resultado del examen de fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	No. 11-009237-00000-0000	27/Enero/2011
Reivindicaciones:	No. 11-009237-00000-0000	27/Enero/2011
	No. 11-009237-00004-0000	19/Mayo/2011
Dibujos:	No. 11-009237-00000-0000	27/Enero/2011
Secuencias:	No. 11-009237-00000-0000	27/Enero/2011
Prioridad:	US 61/083, 699	25/Julio/2008

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque ésta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del capítulo primero del Título 10 de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones

adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada. En el presente caso se estudiará todo el capítulo reivindicatorio No. 11-009237-00004-0000 (14 reivindicaciones).

Título de la solicitud: “La proteína de tuberculosis RV2386C, composiciones y usos de las mismas”.

El problema planteado en esta solicitud consiste en la necesidad de obtener estrategias efectivas para el tratamiento y la prevención de tuberculosis.

Para resolver este problema técnico la solicitud en estudio proporciona un polipéptido que comprende una secuencia de proteína de Rv2386c. También proporciona polinucleótidos, proteínas de fusión, y métodos asociados para el tratamiento o la prevención de la tuberculosis.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): C12N 15/60, A61K38/51; A61K38/52; A61K39/04; C07K14/35; C12N15/61; C12N9/88; C12N9/90.

4. No es una invención (Art 15 D 486 literal (b))

El objeto de las reivindicaciones 1-2 (proteína Rv2386c aislada de Mycobacterium tuberculosis) no se considera una invención, de acuerdo con el Art citado.

5. De la solicitud de patente

Título

El título no corresponde con el objeto de la solicitud porque el uso de la invención no es patentable, por lo cual se sugiere al solicitante hacer la corrección necesaria.

Reivindicaciones (Art 30 D 486, Art 22 PCT)

- 1- El término “fragmento” de las reivindicaciones 3, 5 y 10-14 no es claro. Se sugiere eliminar la mención a fragmentos debido a que no corresponden a estructuras que estén debidamente probadas y sustentadas en la descripción.
- 2- Las reivindicaciones 13 y 14 no son claras debido a que no se especifica la estructura de la secuencia de la proteína Rv2386c, ni las variantes de la proteína Rv2386c, ni la secuencia del fragmento inmunogénico. Se recuerda al solicitante que la reivindicación independiente debe contener todos los elementos esenciales de la invención y debe ser definido a partir de su estructura, una proteína de fusión se define por sus secuencias específicas de aminoácidos. Se sugiere eliminar la mención a las variantes de la proteína Rv2386c debido a que no corresponden a estructuras debidamente probadas y sustentadas en la descripción.
- 3- El capítulo reivindicatorio no es conciso porque contiene un excesivo número de reivindicaciones independientes, lo cual hace difícil definir cuál es el objeto que se busca proteger.
- 4- La composición de las reivindicaciones 3 -9 debe ser caracterizada a partir de los ingredientes que la conforman y las proporciones de los mismos. La variante de la proteína Rv2386 carece de sustento en la descripción. Se sugiere eliminar la mención a las variantes de la proteína Rv2386c debido a que no corresponden a estructuras debidamente probadas y sustentadas en la descripción.

- 5- La reivindicación 10 que se refiere a un vector de ADN recombinante debe ser caracterizado por los elementos que conforman este vector, específicamente por las secuencias de nucleótidos que incluye. Donde las secuencias, la conformación y actividad de estos vectores debe estar completamente sustentada en la descripción.
- 6- La célula huésped, reclamada en las reivindicaciones 11 y 12, debe ser definida como una línea celular y estar sustentada con el número de depósito.
- 7- Las reivindicaciones 5-9 no son claras debido a que no se define la composición de los adyuvantes QS21 y 3DMPL
- 8- La referencia a porcentajes de identidad en las reivindicaciones 7-9 no es aceptable porque no permiten definir la estructura completa del polipéptido y no corresponden a estructuras obtenidas o probadas. Se sugiere eliminar la referencia a los mismos del pliego reivindicatorio.

Se requiere al solicitante hacer las aclaraciones necesarias.

6. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: Julio 25 de 2008, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	Vipond et al., 2006. Vaccine 24 (2006). 6340–6350	“Selection of novel TB vaccine candidates and their evaluation as DNA vaccines against aerosol challenge”	11/Septiembre/2006

El documento D1¹ divulga el uso de ácidos nucleicos codificando la proteína Rv2386c de *Mycobacterium tuberculosis* como una vacuna de ADN. También divulga la expresión de vectores comprendiendo el ácido nucleico, células huésped transformadas y métodos para producir la proteína (Pág. 6341, Col. 2, Tabla 1, Pág. 6342, Pág. 6345, Col. 1).

7. Examen de Patentabilidad (Art 14 D486)

Novedad (Art 16 D486)

La reivindicación 10 consiste en: *“un vector de expresión el cual comprende una secuencia de ácido nucleico que codifica un polipéptido (...)”*

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D1
Vector que comprende	Vector que comprende
Una secuencia de proteína de Rv2386c	Secuencia de proteína de Rv2386c (Tabla 1, Pág. 6342, Pág. 6341, Col. 2, Párr. 3)

Las características esenciales de la reivindicación 10 habían sido divulgadas previamente en el documento D1, el cual da a conocer la expresión de vectores comprendiendo el ácido nucleico (Pág. 6341, Col. 2, Párr. 3, Pág. 6342, Tabla 1).

En consecuencia, la reivindicación 10 no es nueva o carece de novedad, según lo divulgado en el documento D1.

La reivindicación 11 consiste en: *“una célula huésped aislada que expresa de una manera recombinante un polipéptido (...)”*

1 . <http://dx.doi.org/10.1016/j.vaccine.2006.05.025>

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D1
Célula huésped aislada que comprende	Célula huésped (Pág. 6345, Col. 1, Párr. 3)
Una secuencia de proteína Rv2386c	Secuencia de proteína Rv2386c (Tabla 1, Pág. 6342)

Las características esenciales de la reivindicación 11 habían sido divulgadas previamente en el documento D1, el cual da a conocer ácidos nucleicos codificando la proteína Rv2386c de *Mycobacterium tuberculosis* (Tabla 1, Pág. 6342) y células huésped transformadas (Pág. 6345, Col.1, Párr. 3).

En consecuencia, la reivindicación 11 no es nueva o carece de novedad, según lo divulgado en el documento D1.

La reivindicación dependiente 12 no menciona ninguna característica que haga nueva a la célula huésped por lo cual carece de novedad.

La reivindicación 13 consiste en: *“una proteína de fusión, la cual comprende un polipéptido que comprende (...)”*.

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D1
Proteína de fusión	Proteína de fusión
Polipéptido que comprende una secuencia de proteína Rv2386c	Fusión del gen de la proteína Rv2386c con el gen que codifica la secuencia señal del activador del tejido humano plasminogen (Pág. 6345, Col. 1, Párr.2)

Las características esenciales de la reivindicación 13 habían sido divulgadas previamente en el documento D1, el cual da a conocer ácidos nucleicos codificando la proteína Rv2386c de *Mycobacterium tuberculosis*, ácidos nucleicos que codifican la fusión del gen que codifica la proteína Rv2386c con el gen que codifica la secuencia señal del activador del tejido humano plasminogen (Pág. 6345, Col. 1, Párr.2).

En consecuencia, la reivindicación 13 no es nueva o carece de novedad, según lo divulgado en el documento D1.

La reivindicación 14 consiste en: *“un polinucleótido que codifica una proteína de fusión, la cual comprende (...)”*.

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D1
Polinucleótido que codifica una proteína de fusión que comprende	Acido nucleico que codifica
Polipéptido que comprende una secuencia de proteína Rv2386c	Fusión del gen codificando la proteína Rv2386c con el gen que codifica la secuencia señal del activador del tejido humano plasminogen (Pág. 6345, Col. 1, Párr.2)

Las características esenciales de la reivindicación 14 habían sido divulgadas previamente en el documento D1, el cual da a conocer ácidos nucleicos codificando la proteína Rv2386c de *Mycobacterium tuberculosis*, ácidos nucleicos que codifican la fusión del gen que codifica la proteína Rv2386c con el gen que codifica la secuencia señal del activador del tejido humano plasminogen (Pág. 6345, Col. 1, Párr.2).

En consecuencia, la reivindicación 14 no es nueva o carece de novedad, según lo divulgado en el documento D1.

Nivel inventivo (Art18 D486)

La reivindicación 3 consiste en: “una composición la cual comprende: a. un polipéptido b. un polinucleótido, c. un potenciador de respuesta inmunitaria (...)”

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D1
Composición que comprende	Vacuna (Pág. 6341)
Polipéptido que comprende una secuencia de proteína de Rv2386c, una variante de una secuencia de proteína de Rv2386c, un fragmento inmunogénico	Polipéptido que comprende una secuencia de proteína de Rv2386c (Tabla 1, Pág. 6342)
Polinucleótido que comprende una secuencia de ácido nucléico	Polinucleótido que comprende una secuencia de ácido nucleico (Pág. 6341)
Potenciador de respuesta inmunitaria no específica	No menciona

El documento D1 se considera el estado de la técnica cercano a la invención definida en la reivindicación 3 porque divulga uso de ácidos nucleicos codificando la proteína Rv2386c de *Mycobacterium tuberculosis* como una vacuna de ADN (Resumen).

La diferencia entre la invención, definida en la reivindicación 3 y la vacuna de D1 consiste en el potenciador de respuesta inmunitaria no específica.

El efecto que logra el incluir el potenciador de la respuesta inmunitaria no específica es que inducen la respuesta inmunitaria no específica.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: “cómo modificar la vacuna conocida en D1 para conseguir una vacuna alternativa que aumente la respuesta inmunitaria contra la tuberculosis”.

Sin embargo, la utilización del DNA codificando Rv2386c como una vacuna, ya está divulgada en el documento D1 que se refiere a vacunas para la protección de *Mycobacterium tuberculosis* (Resumen) que inducen la mediación de la respuesta inmune.

Teniendo en cuenta que el documento D1 ya divulgaba vacunas para el tratamiento de la tuberculosis usando ácidos nucleicos que codifican la proteína Rv2386c, la persona del oficio medianamente versada en la materia habría inferido sin mucho esfuerzo de su parte, generar vacunas alternativas que inducen la respuesta inmunitaria no específica para el tratamiento de la tuberculosis. Por lo cual la reivindicación 3 se considera obvia.

Las reivindicaciones dependientes 4-9 no cumplen con el requisito de nivel inventivo.

8. Conclusión

Los requisitos de Patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	Vipond et al., 2006. Vaccine 24 (2006) 6340–6350	10-14	Novedad
D1	Vipond et al., 2006. Vaccine 24 (2006) 6340–6350	3-9	Nivel Inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2013-09-10

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Selection of novel TB vaccine candidates and their evaluation as DNA vaccines against aerosol challenge

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Abstract

Putative TB vaccine candidates were selected from lists of genes induced in response to *in vivo*-like stimuli, such as low oxygen and carbon starvation or growth in macrophages, and tested as plasmid DNA vaccines for their ability to protect against *Mycobacterium tuberculosis* challenge in a guinea pig aerosol infection model. This vaccination method was chosen as it induces the Th1 cell-mediated immune response required against intracellular pathogens such as *M. tuberculosis*. Protection was assessed in the guinea pig model in terms of mycobacteria present in the lungs at 30 days post-challenge. Protection achieved by the novel candidates was compared to BCG (positive control) and saline (negative control). Four vaccines encoding for proteins such as PE and PPE proteins, a zinc metalloprotease and an acyltransferase, gave a level of protection that was statistically better than saline in the lungs. These findings have enabled us to focus on a sub-set of vaccine candidates for further evaluation using additional vaccination strategies.

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Keywords: *Mycobacterium tuberculosis*; DNA vaccine; Guinea pig

1. Introduction

Currently, the only available vaccine against tuberculosis (TB) is bacillus Calmette-Guérin (BCG) and has been received by over three billion people worldwide [1]. Although BCG is most effective in protecting children from the disease, its efficacy varies globally, ranging from 0% to 80% [2], so

that the development of an improved vaccine is a research priority [3].

The past two decades have seen a significant increase in the development of potential new vaccines for tuberculosis, due mainly to advances in the discovery and characterisation of *Mycobacterium tuberculosis* virulence factors, aided by the publication of the complete TB genome sequence [4]. Several methods of vaccine delivery have been used in pre-clinical screening of TB vaccines with no particular delivery route or formulation being favoured [5,6]. One method of delivering sub-unit vaccines is the direct delivery of DNA encoding the gene of interest. Here the genes encoding the protein antigens are delivered into host cells thereby enabling antigen production to occur *in vivo*. DNA vaccines stimulate both the MHC Class II restriction and MHC Class I restricted antigen presentation pathways, and the strong

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CD8⁺ T cell response resulting from the MHC Class I presentation is a characteristic of DNA vaccines [7]. DNA vaccines have been used as a successful approach for several TB candidates [8–11] and are likely to remain a common formulation method for at least the initial evaluation of vaccine potential, especially with their ease of preparation and stability, plus the adjuvant effect that they elicit upon delivery. To enhance DNA vaccination, plasmids can encode for a secreted form of the protein by fusing the gene to the signal sequence of human tissue plasminogen activator (tPA), often including the 12-residue pro-sequence [12–15]. These are often more immunogenic than plasmids purely encoding the mature form of the protein [16] and inclusion of the pro-sequence is thought to enhance intracellular transport [17].

Two of the main models used for preclinical TB vaccine screening are the low-dose aerosol mouse and guinea pig models. The majority of the work has been carried out in various mouse models due to their low cost and the wealth of immunological reagents. However, there is an increased knowledge and availability of the guinea pig infection model of tuberculosis and new immunological reagents are continuously being developed, thus leading to an increase in vaccine testing in the guinea pig model [6,18–24]. Aerosol infection of guinea pigs with a low-dose of *M. tuberculosis* results in a progression of disease that is similar to that seen in humans [25]. The difference between vaccinated and unvaccinated guinea pigs tends to be greater than similarly treated mice and thus the guinea pig model is more discriminative than mice for vaccine evaluation [23]. Guinea pigs also develop delayed-type hypersensitivity (DTH) reactions that histologically closely resemble those of humans.

An effective sub-unit vaccine would have many advantages over a live, attenuated vaccine such as BCG, and in this study we used two complementary approaches to identify genes required for infection that could subsequently be used as vaccines. Differential fluorescence induction (DFI) was used to reveal genes expressed during mycobacterial growth in macrophages [26], while microarray and transcriptome analyses were undertaken on cells grown under defined and controlled conditions reflecting those likely to be encountered in the host [27,28].

In this study we evaluated DNA vaccines encoding selected genes for their ability to protect guinea pigs against a low-dose of *M. tuberculosis* delivered via the aerosol route. Candidates selected from those identified by DFI or controlled growth conditions were compared against the current vaccine, BCG and a saline control. Vaccine candidate efficacy in guinea pigs was assessed on the basis of reducing the bacterial burden of *M. tuberculosis* in the lungs at 30 days post-aerosol challenge. We report here that four of the selected genes were able to afford protection that was significantly better than the negative control group on two repeat experiments.

2. Materials and methods

2.1. Bacterial strains and plasmids

Escherichia coli strain DH5 α was cultured in Luria-Bertani medium as previously described [29]. Plasmid pVAX1 and *Pfx* DNA polymerase were obtained from Invitrogen, Paisley, UK. Endotoxin-free plasmid purification kits were used according to the manufacturer's instructions (Qiagen, Crawley, UK).

2.2. Selection of vaccine candidates

Lists of *M. tuberculosis* genes (data not shown) induced during infection of macrophages, were generated using differential fluorescence induction (DFI) as a silent marker of gene expression in a model of infection [26]. Briefly, *M. tuberculosis* H37Rv was transformed with a plasmid promoter library and a macrophage infection assay performed using J774A.1 cells (ECACC, Salisbury, UK). Multiple rounds of differential screening using a CL3-contained FACS sorter (Becton-Dickinson, New Jersey, USA) enabled induced promoters to be identified following sequencing of recovered plasmids from cell-sorted *M. tuberculosis* [30]. Genes that were up- or down-regulated in response to relevant environmental stresses, as determined by DNA microarray analysis, were selected from either an oxygen-depleted continuous culture [27] or a carbon-depleted stationary-phase model [28] of *M. tuberculosis*, respectively. Briefly, a sample of RNA was extracted from four independent chemostat cultures grown under both oxygen-replete and oxygen-depleted conditions, and from one culture grown under carbon-depleted conditions, and then used for DNA microarray as described previously [27,28]. Genes showing statistically significant changes in expression level were selected by at least a 1.5-fold change in expression combined with Welch's approximate *t*-test and a *P*-value cut off of 0.05. Expression levels of at least 5-fold higher or lower at day 50 relative to day 5 were used for those genes identified from the stationary-phase culture. The analysis of expression levels was performed using GeneSpring software and information can be found at the Agilent Technologies website [31]. Selection criteria (e.g., novel, secretory, homology to known virulence factors and predicted expression level) were applied to these lists to identify a sub-set of genes that were likely to provide novel vaccine candidates (Table 1).

2.3. DNA vaccine vector construction

Standard molecular biology techniques were used throughout [29]. The plasmid pVAX1 was designed in accordance with the FDA guidelines for DNA vaccine vectors. The basic vector containing the cytomegalovirus (CMV) immediate/early promoter and bovine growth hormone (bgh) polyadenylation sequence, was modified by the introduction of linkers encoding the signal peptide and pro-sequence

Table 1

Selected vaccine candidates with their predicted functions and conditions from which they were identified

Group number	Vaccine candidate	H37Rv gene	Predicted function	Identifying growth condition
1	1	Rv1477	Putative exported p60 homologue	DFI ^a
		Rv1478	Hypothetical Invasion protein	
	2	Rv1994c	Transcriptional regulatory protein	Up-regulation under low oxygen
	3	Rv1919c	Conserved hypothetical protein	DFI
	4	Rv3174	Probable short-chain dehydrogenase	Up-regulation under low oxygen
	5	Rv3402c	Conserved hypothetical protein	Up-regulation under low oxygen
	6	Rv2462 <i>tig</i>	Probable trigger-factor protein	DFI
2	7	Rv1344	Probable acyl-carrier proteins	Up-regulation under low oxygen
		Rv1345 <i>fadD33</i>	Possible polyketide synthase	
		Rv1346 <i>fadE14</i>	Possible acyl Co-A dehydrogenase	
	8	Rv1130	Conserved hypothetical protein	Up-regulation under low oxygen
		Rv1131 <i>glcA1</i>	Probable citrate synthase	
		Rv1132	Conserved membrane protein	
	9	Rv2386c <i>mbtI</i>	Putative isochorismate synthase	Up-regulation under low oxygen
	10	Rv0251c <i>hsp</i>	Heat shock protein	Up-regulation under low oxygen and carbon starvation
	11	Rv0384c <i>clpB</i>	Probable ATP-binding protein	DFI
	12	Rv1179c	Hypothetical protein	DFI
3	13	Rv0111	Acyltransferase	Up-regulation under carbon starvation and high oxygen
	14	Rv1646 PE17	PE protein	DFI
	15	Rv1799 <i>lppT</i>	Probable lipoprotein	Up-regulation under high oxygen
	16	Rv1917c PPE34	PPE protein	Up-regulation under carbon starvation and high oxygen
	17	Rv1909c <i>furA</i>	Ferric uptake regulatory protein	DFI
		Rv1910c	Probable exported protein	
		Rv1911c <i>lppC</i>	Probable lipoprotein	
		Rv1912c <i>fadB5</i>	Possible oxidoreductase	
	18	Rv0913c	Possible dioxygenase	Up-regulation under carbon starvation and high oxygen
		Rv0914c	Possible lipid carrier protein	
		Rv0915c PPE14	PPE family protein	
		Rv0916c PE7	PE family protein	

Table 1 (Continued)

Group number	Vaccine candidate	H37Rv gene	Predicted function	Identifying growth condition
4	19	Rv2671 <i>ribD</i>	Possible riboflavin biosynthesis protein	DFI
		Rv2672	Possible secreted protease	
		Rv2673	Possible conserved integral membrane protein	
		Rv2037c	Possible transmembrane protein	DFI and up-regulation under carbon starvation
	20	Rv2038c	Probable sugar-transport ATP-binding protein	
		Rv2039c	Probable sugar-transport integral ABC transporter	
		Rv2040c	Probable sugar-transport integral ABC transporter	
		Rv2041c	Probable sugar-binding lipoprotein	
	21	Rv1167c	Probable regulatory protein	
		Rv1168c PPE17	PPE family protein	Up-regulation under low oxygen
		Rv1169c PE11	PE family protein	
	22	Rv0785	Conserved hypothetical protein	Up-regulation under carbon starvation
	23	Rv3639c	Conserved hypothetical protein	Up-regulation under low oxygen
	24	Rv0847 <i>lpqS</i>	Probable lipoprotein	Up-regulation under low oxygen
	25	Rv0395	Hypothetical protein	Up-regulation under carbon starvation and low oxygen
		Rv0396	Hypothetical protein	
		Rv0397	Conserved repeat family protein	
5	26	Rv3444c <i>esxT</i>	Putative ESAT-6 like protein	
		Rv3445c <i>esxU</i>	Putative ESAT-6 like protein	
		Rv3446c	Hypothetical alanine and valine rich protein	Up-regulation under low oxygen
		Rv3447c	Probable conserved membrane protein	
	27	Rv2316 <i>uspA</i>	Probable ABC sugar transport	Up-regulation under high oxygen
		Rv2317 <i>uspB</i>	Probable sugar transport membrane protein	
		Rv2318 <i>uspC</i>	Probable periplasmic sugar-binding lipoprotein	
	28	Rv0816c <i>thiX</i>	Probable thioredoxin	Up-regulation under high oxygen
		Rv0817c	Probable conserved exported protein	
	29	Rv2667 <i>clpC2</i>	Possible ATP-dependent protease	Up-regulation under high oxygen
		Rv2668	Possible exported alanine and valine rich protein	
		Rv2669	Conserved hypothetical protein	

Table 1 (Continued)

Group number	Vaccine candidate	H37Rv gene	Predicted function	Identifying growth condition
6	30	Rv1840c	PE-PGRS protein	Up-regulation under low oxygen
		Rv1841c	Conserved hypothetical membrane protein	
		Rv1842c	Conserved hypothetical membrane protein	
	31	Rv0198c	Probable zinc metalloprotease	Down-regulation under carbon starvation
	32	Rv0228	Probable acyltransferase	Up-regulation under carbon starvation
	33	Rv2999 <i>lppY</i>	Probable conserved lipoprotein	Up-regulation under low oxygen
		Rv3000	Conserved hypothetical membrane protein	
	34	Rv1806 PE20	PE protein	Up-regulation under carbon starvation
	35	Rv1807 PPE31	PPE family protein	Up-regulation under low oxygen
		Rv3812 PE.PGRS62	PE-PGRS protein	
	36	Rv1228 <i>lpqX</i>	Probable lipoprotein	Up-regulation under low oxygen
	37	Rv2516c	Hypothetical protein	Up-regulation under low oxygen
7	38	Rv0544c	Possible conserved transmembrane protein	Down-regulation under carbon starvation
		Rv0545c <i>pitA</i>	Probable low affinity inorganic phosphate transporter	
	39	Rv1342c	Conserved membrane protein	Up-regulation under low oxygen
		Rv1343c <i>lprD</i>	Probable conserved lipoprotein	
	40	Rv3756 <i>proZ</i>	Possible osmoprotectant	Up-regulation under low oxygen
		Rv3757 <i>proW</i>	Possible osmoprotectant	
		Rv3758 <i>proV</i>	Possible osmoprotectant	
		Rv3759 <i>proX</i>	Possible osmoprotectant	
	41	Rv2896c	Conserved hypothetical protein	Up-regulation under high oxygen
		Rv2897c	Conserved hypothetical protein	
		Rv2898c	Conserved hypothetical protein	
8	42	Rv1761c	Hypothetical exported proteins	Up-regulation under high oxygen
		Rv1762c	Hypothetical protein	
	43	Rv0848 <i>cysK2</i>	Possible cysteine synthase	Up-regulation under carbon starvation
		Rv0849	Probable conserved integral membrane transport protein	
	44	Rv0917 <i>betP</i>	Glycine betane transporter	Up-regulation under carbon starvation
	45	Rv1633 <i>uvrB</i>	Probable exonuclease	Up-regulation under low oxygen
		Rv1634	Possible drug efflux membrane protein	
	46	Rv2144c	Probable transmembrane protein	Down-regulation under carbon starvation
		Rv2145c <i>wag31</i>	Conserved hypothetical protein	

Table 1 (Continued)

Group number	Vaccine candidate	H37Rv gene	Predicted function	Identifying growth condition
47		Rv2460c <i>clpP2</i>	Probable ATP-dependent <i>clp</i> protease	DFI
		Rv2461c <i>clpP1</i>	Probable ATP-dependent <i>clp</i> protease	

Where an identified gene formed an operon then each gene within that operon was represented in the vaccine with an equal concentration of DNA. Vaccines were evaluated in eight independent experiments, designated groups 1–8.

^a Differential fluorescence induction, used to identify genes expressed during growth in macrophages.

of tPA to form pVAX1tPA. To allow studies on candidate expression *in vitro*, a linker encoding a 6-His tag was inserted into the vector to form the derivative pVAX1tPAHIS. PCR amplification of the gene encoding eGFP (Clontech, Oxford, UK) and ligation of the amplified fragment into pVAX1tPAHIS created a second *in vitro* expression vector pVAX1tPAeGFPHIS. All vectors contained in-frame cloning sites with compatible cohesive ends corresponding to those introduced into the ends of cloned antigens by PCR; these were AT-rich, and thus rare (*Hind*III) or absent (*Pac*I) in the *M. tuberculosis* genome.

2.4. DNA vaccine construction

Selected vaccine candidate genes were screened for the presence of a predicted signal peptide using SignalP [32]. All of the genes were amplified from *M. tuberculosis* H37Rv chromosomal DNA using *Pfx* polymerase (Invitrogen), using primers designed from the *M. tuberculosis* genome sequence database. Primers incorporated restriction sites to allow in-frame fusion to the tPA signal peptide/pro sequence at the start of the mature gene sequence (minus the start codon) and the 6-His tag at the carboxy terminus where required. PCRs were carried out using cycle conditions of 95 °C, 1 min, followed by 30 cycles of 95 °C, 15 s, 60 °C, 15 s, 68 °C, 15 s, followed by a final extension of 68 °C, 7 min. PCR amplified genes were cloned into pVAX1tPA. Selected clones were verified by sequencing, following analysis by restriction digestion. The sequenced gene fragments were sub-cloned into a related vector for *in vitro* studies (pVAX1tPAeGFPHIS). Large-scale endotoxin-free preparations of plasmid (Qiagen) were then made to obtain plasmid DNA at 1 mg/ml (total of 6 mg per candidate). Where the vaccine candidate was comprised of more than one gene (e.g., an operon such as vaccine candidate 7, Table 1) then each gene within that operon was represented in the vaccine with an equal concentration of plasmid DNA.

2.5. *In vitro* expression

Vaccine candidates were sub-cloned into pVAX1tPAeGFPHIS and transfected into COS-7 cells (ECACC, Salisbury, UK) grown on poly-D-lysine coated cover slips

(BD Bioscience) using Lipofectamine 2000™ (Invitrogen). COS-7 cells were maintained in DMEM (supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 10% foetal calf serum and 2 mM glutamine) at 37 °C and 5% CO₂ for 24 h. Cells were viewed 24 h post-transfection using a FITC filter on a fluorescence microscope at a wavelength of 460–500 nm.

2.6. Vaccination and aerosol infection of guinea pigs

Female Dunkin–Hartley guinea pigs (David Hall, Burton-on-Trent, UK) were vaccinated with plasmid DNA constitutively expressing selected TB genes. Briefly, 100 µg of DNA was injected intramuscularly into both hind quadriceps muscles of each guinea pig (six animals per group). As it was not possible to evaluate all 47 candidates in 1 experiment, they were divided into 8 groups for evaluation (generally 6 candidates plus controls). Each vaccination group also contained both positive (BCG) and negative (saline) controls against which the vaccine candidates were assessed. One group also included an empty vector control to ensure there were no non-specific mechanisms of protection. The six positive control animals were inoculated sub-cutaneously with 5×10^4 cfu BCG Pasteur, and six negative control animals received saline. DNA-vaccinated animals received booster vaccinations as above after 3 and 6 weeks. Six weeks after the final vaccination animals were aerosol challenged, using a contained Henderson apparatus with *M. tuberculosis* H37Rv (NCTC 7416) to give a retained dose in the lung of approximately 20–50 cfu per animal [33].

Protection was assessed 30 days post-challenge as described previously [33]. Lung tissue was aseptically removed post-mortem and homogenised in 10 ml sterile distilled water. Ten-fold dilutions of these samples were performed (10^{-1} , 10^{-2} , 10^{-3}) and plated onto Middlebrook 7H11 plus OADC selective agar. Plates were incubated at 37 °C and examined after 3 weeks for growth of *M. tuberculosis*. Vaccine efficacy was assessed in terms of reduction in bacterial counts in lungs compared to the saline control group. The mean log₁₀ cfu of test vaccines were compared with the negative controls and differences analysed statistically (see below).

2.7. Statistical analysis

Values for the mean and standard error of the mean were calculated for each data set. Data was analysed both by the Student's *t*-test and ANOVA, using Fisher's pair-wise comparisons to compare the mean values of all the groups in the experiments. Probability values of <0.05 were taken as significant and promising candidates were re-assessed in repeat groups.

3. Results

3.1. Vaccine candidate selection

M. tuberculosis genes initially identified as possible vaccine candidates, included (i) those genes that were induced in response to infection and growth in macrophage (identified by DFI [26,30]), and (ii) those that were up- or down-regulated in response to oxygen and carbon limitation (identified by DNA microarray analysis of mRNA isolated from cultures [27,28]). Lists of genes were then subjected to further selection to reduce the number of candidates. The selection criteria against which the candidates were chosen were:

- (1) Novel: to our knowledge, all candidates that were selected were not patented, published or being investigated by others as a TB vaccine at the time of the study.
- (2) Secretory: secreted antigens were considered to make better candidates for activation of both CD4⁺ and CD8⁺ T cells, thereby explaining why the majority of immunogenic TB antigens are those present in the culture filtrate of *M. tuberculosis*. This was investigated by the use of SignalP (a program that predicts the presence and location of signal peptide cleavage sites based on a combination of several neural networks and hidden Markov models) [32]. Approximately 60% of selected candidates were predicted to be secreted using this approach.
- (3) Supporting data: if the candidate was identified from more than one growth condition then there was more sup-

porting data for its inclusion as a vaccine candidate (six candidates met this criterion). Also, a focus on candidates that had a virulence-associated function, or homology to known virulence factors, increased the chances of the candidate having a potential role in infection.

- (4) Predicted expression levels: where available, published data detailing predicted gene expression levels, indicating genes that are highly expressed, were used to select several candidates (data not shown) [34–36].

Ultimately 47 vaccine candidates (comprising 92 genes) were selected using the above criteria from the lists of DNA microarray analysis of cultures grown under defined conditions and candidates identified by DFI (Table 1).

3.2. Construction of pVAX1tPA and pVAX1tPAeGFPHis vectors

Fig. 1 details pVAX1tPA showing the orientation of the CMV promoter (*P_{CMV}*), tPA signal peptide/pro-sequence (*tPA*), restriction cloning site (RCS) and bgh polyadenylation sequence (*bgh*). Plasmid pVAX1tPAeGFPHis shows the cloned His tag (His) and the *eGFP* fragment used for immunofluorescence detection. DNA fragments of the selected vaccine candidates obtained by PCR were digested with each restriction endonuclease and ligated into *Pac*I- and *Hind*III-digested plasmid pVAX1tPA. The resultant clones were confirmed by restriction analysis and DNA sequencing (data not shown).

3.3. In vitro expression

Positively identified vaccine clones were sub-cloned into pVAX1tPAeGFPHis to evaluate expression *in vitro* prior to vaccination. COS-7 cells (ECACC, Salisbury, UK) were transfected with the positive clones using Lipofectamine 2000TM (Invitrogen). eGFP fluorescence in COS-7 cells transfected with the positive vaccine clones confirmed the ability of the plasmid to produce the protein. All genes were shown to be expressed prior to vaccination (data not shown).

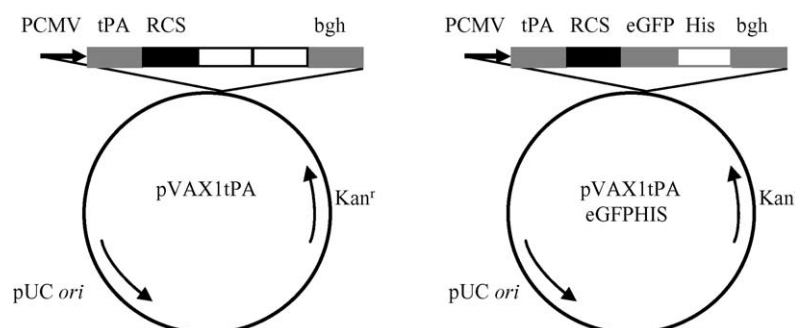


Fig. 1. Schematic diagram of the various forms of pVAX1tPA showing orientation of the CMV promoter (*P_{CMV}*), tPA signal peptide/pro-sequence (*tPA*), restriction cloning site (RCS) and bgh polyadenylation sequence (*bgh*). Plasmid pVAX1tPAeGFPHis shows the cloned eGFP fragment used for immunofluorescence detection.

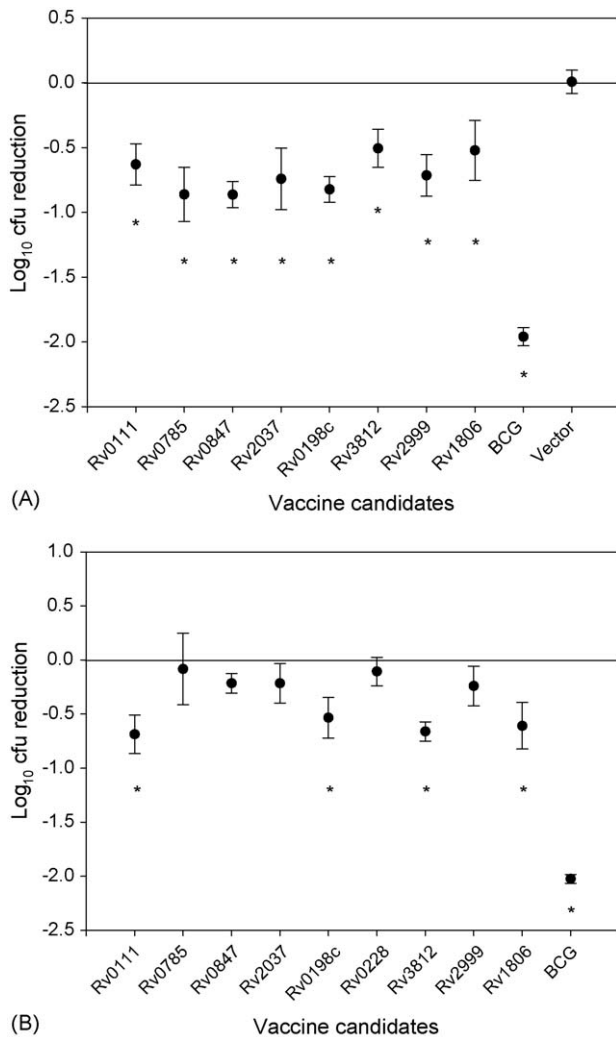


Fig. 2. (A) Bacterial log₁₀ reduction in the lungs, compared to saline control, as a marker of DNA vaccine candidate efficacy following aerosol challenge with *M. tuberculosis* H37Rv. (B) Bacterial log₁₀ reduction following a repeat challenge experiment. The bars indicate the mean $\pm$ S.E. ($n=6$), * $P<0.05$ (Student's t -test).

3.4. Protection against *M. tuberculosis* aerosol challenge

To evaluate any potential protective effect of the candidate vaccines, groups of six guinea pigs received three DNA vaccinations followed by an aerosol challenge of H37Rv at week 15. Fig. 2(A) shows log₁₀ cfu reduction from saline in lungs of animals challenged with virulent *M. tuberculosis* by the aerosol route. The antigens used for vaccination are indicated and the results are the mean $\pm$ S.E. from groups of six guinea pigs. Also included in the evaluation was an empty vector control group to ensure there was no non-specific protection. The experiment was repeated with the DNA vaccine candidates that showed statistically significant cfu reduction in the lungs compared to saline, and these are shown in Fig. 2(B).

In summary, none of the candidates protected better than or as well as BCG, although eight candidates gave a level

of protection that was better than the saline or vector control groups in the lungs on the first evaluation. Four of these candidates (Rv0111, Rv0198c, Rv3812 and Rv1806–1807) displayed protection that was statistically better than saline, by the Student's t -test ($p<0.05$) and ANOVA, after the second repeat experiment (Fig. 2 and Table 2), but there was no statistically significant difference between these candidates.

4. Discussion

Complementary approaches have been used to identify genes that were expressed or up- or down-regulated under conditions that *M. tuberculosis* might encounter in the host and which could, therefore, be considered as potential vaccine candidates. DFI was used as a silent marker of gene expression to reveal TB genes induced during uptake and replication in the macrophage, a key step in the infection process, while the controlled environment culture studies enabled us to grow *M. tuberculosis* in a defined and virulent physiological state under conditions relevant to the host (low oxygen [27], carbon starvation [28]). The strategy for identifying putative protective antigens from the gene lists generated from the DFI studies and from microarray analysis of extracted RNA, was based upon specific selection criteria (e.g., proteins likely to be secreted and thus more accessible to the immune system, genes that were identified by more than one approach, genes that were novel, and any additional supporting data such as putative virulence-associated functions). This enabled a short-list of 47 candidates to be drawn up for evaluation out of a total of several hundred genes. Lists of genes have also been generated by others studying the gene expression profile of *M. tuberculosis* [37,38]. In one of these studies, where changes in the expression profile of H37Rv and a *sigH* mutant were examined in response to the oxidising agent, diamide, two of our vaccine candidates were identified. Rv0251c was shown to be induced due to this general stress response in a *sigH*-independent manner and Rv1130 in a *sigH*-dependent manner [37]. This provided subsequent confirmatory data (to our finding of induction under low oxygen and carbon starvation) that these genes are involved in the response to stresses that may be encountered *in vivo* and therefore could be relevant targets for a directed immune response. The selected 47 candidates were systematically evaluated for their ability to protect guinea pigs against a low-dose of *M. tuberculosis* compared with BCG and a saline control.

There was no obvious relationship between the putative functions of the four candidates that were protective in both the first and second rounds of evaluation. Three of the candidates, PE and PPE proteins (Rv1806–1807, Rv3812) and an acyltransferase (Rv0111), were up-regulated under conditions of stress that may be encountered in the host (oxygen and carbon starvation) and were thought to be important in the stress response or in persistence. Approximately 10% of the *M. tuberculosis* genome is allocated to the PE and PPE gene families, and although their exact function is unknown,

Table 2

Viable *M. tuberculosis* log₁₀ cfu counts in the lungs from guinea pigs vaccinated with DNA vaccines compared to unvaccinated (saline) animals and BCG-vaccinated animals relevant to each experiment group

Vaccine	First evaluation log ₁₀ cfu	Log ₁₀ reduction ^a	Second evaluation log ₁₀ cfu	log ₁₀ reduction ^a
BCG ^b	3.79 ± 0.12	2.11	3.23 ± 0.14	2.01
Saline	5.90 ± 0.21	0.00	5.24 ± 0.08	0.00
Rv0111 ^c	5.21 ± 0.11	0.69	4.55 ± 0.17	0.69
BCG ^b	3.49 ± 0.34	1.77	3.45 ± 0.32	1.96
Saline	5.26 ± 0.33	0.00	5.41 ± 0.20	0.00
Rv0785	4.41 ± 0.20	0.85	5.33 ± 0.33	0.08
Rv0847	4.40 ± 0.10	0.86	5.20 ± 0.09	0.21
Rv2037	4.52 ± 0.23	0.74	5.20 ± 0.18	0.21
BCG ^b	3.23 ± 0.14	2.01	3.45 ± 0.10	2.1
Saline	5.24 ± 0.08	0.00	5.55 ± 0.20	0.00
Rv0198 ^c	4.42 ± 0.10	0.82	5.02 ± 0.19	0.53
Rv3812 ^c	4.73 ± 0.14	0.51	4.89 ± 0.09	0.66
Rv2999	4.53 ± 0.16	0.71	5.31 ± 0.18	0.24
Rv1806 ^c	4.72 ± 0.23	0.52	4.94 ± 0.21	0.61

The values given are means (±S.E.) (*n* = 6) for both the first and second evaluations.

^a log₁₀ reduction in cfu compared to saline.

^b *p* < 0.01 for first and second evaluations.

^c *p* < 0.05 for first and second evaluations.

a similar example has been observed where unknown surface antigens of other pathogens that are targets for the host's immune response have been used as vaccine candidates [39]. Interestingly, TB patients have been shown to mount an immune response to PE proteins, in particular Rv1759 and Rv3367 [7,36], and a significant proportion of BCG-vaccinated donors have been found to recognise the PPE protein, Rv3873 [40]. Mice have also been shown to produce antibodies to the PE_{GRS} protein encoded by Rv1818c following an aerosol infection with *M. tuberculosis* [41]. These data indicate that at least some of the genes belonging to this family are expressed by *M. tuberculosis* *in vivo* and could be recognised by the host immune response. Although there is no supporting evidence for the use of an acyltransferase as a vaccine candidate, other than the consistent results we obtained, such a protein may play a critical role in enzyme activity, such as in lipid metabolism and cell wall biosynthesis. We are currently trying to establish a guinea pig model of chronic infection, aimed at simulating latency, and studies to identify whether Rv0111 is expressed in acute or chronically infected guinea pigs are underway. The fourth candidate, a zinc metalloprotease (Rv0198c), was selected based on its possible virulence-associated function. Unlike the other candidates, this gene was down-regulated under carbon starvation. This may reflect a shift in expression towards a general stress response, whereby the bacterium is tightly regulating its virulence factors and expressing them only when required, and in this instance, not during carbon starvation. Zinc metalloproteases are widely distributed in both Gram positive and Gram negative bacteria, and although a role as a virulence factor in *M. tuberculosis* has not been identified, they have been proposed as a major virulence determinant in several pathogens, e.g. *Burkholderia cenocepacia*, *Clostridium botulinum* and *Bacillus anthracis* [42–45].

Many TB vaccine candidates have been identified using the mouse challenge model; however, some of these candidates are not necessarily protective when they are progressed through the hierarchy of animal models, in particular the guinea pig [18,21,46–48]; a notable exception to this is *M. tuberculosis* 72 kDa fusion polypeptide, where survival in the guinea pig has been shown to be comparable with BCG [49,50]. The guinea pig is regarded as a more stringent model of human tuberculosis than the mouse model and for this reason it is widely used to evaluate new vaccines. Consequently, we chose to screen candidates for protective efficacy directly in the guinea pig aerosol infection model. This strategy of using a more discriminatory screening model, together with our selection criteria, enabled us to reduce the number of candidates to be evaluated to a manageable number and to identify four that afforded a significant and reproducible level of protection. The levels of protection seen in our study are comparable to other DNA vaccines tested in the guinea pig model (e.g. Ag85A and Mtb72f), with a cfu log reduction of between 0.5 and 1.0 [18,50,51]. Sub-unit vaccines (delivered as either protein adjuvant or naked DNA) generally afford lower and rarely equal protection to BCG against challenge with *M. tuberculosis* in guinea pigs; however, prime-boost strategies are able to enhance the level of protection. In these strategies DNA can be used to prime the immune response, then a second vaccination is used to induce a greater response, or BCG can be used as the prime followed by a sub-unit boost [52]. The regimen of DNA as a prime for the Th1 and MHC Class I type immune response, followed by a subsequent protein boost has been used successfully with HIV and malaria [53,54]. Thus, the prime-boost regimen may be the most effective approach in the future for DNA vaccination, particularly in larger animal models.

In conclusion, we have demonstrated the success of using our selection process coupled with the more stringent guinea pig model to move from lists of several hundred potential vaccine candidates down to four that afforded a significant level of protection. We have shown that a plasmid-based system is ideal for screening large numbers of candidate vaccines with respect to relative ease of preparation, cost and time; however, DNA vaccination is likely to be more effective in larger animal models and humans when administered as a prime-boost vaccination regimen, or when given as a combination approach with BCG. These delivery approaches will be used to further evaluate a selection of the protective candidates identified in this study.

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References

- [1] Andersen P, Doherty TM. TB subunit vaccines-putting the pieces together. *Microbes Infect* 2005;7(5–6):911–21.
- [2] Fine PE. Variation in protection by BCG: implications of and for heterologous immunity. *Lancet* 1995;346(8986):1339–45.
- [3] McMurray DN. Recent progress in the development and testing of vaccines against human tuberculosis. *Int J Parasitol* 2003;33(5–6):547–54.
- [4] Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, et al. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* 1998;393(6685):537–44.
- [5] Brennan MJ. The tuberculosis vaccine challenge. *Tuberculosis (Edinburgh)* 2005;85(1–2):7–12.
- [6] Izzo A, Brandt L, Lasco T, Kipnis A-P, Orme I. NIH pre-clinical screening program: overview and current status. *Tuberculosis (Edinburgh)* 2005;85(1–2):25–8.
- [7] Doria-Rose NA, Haigwood N. DNA vaccine strategies: candidates for immune modulation and immunization regimens. *Methods* 2003;31(3):207–16.
- [8] Tascon RE, Colston MJ, Ragno S, Stavropoulos E, Gregory D, Lowrie DB. Vaccination against tuberculosis by DNA injection. *Nat Med* 1996;2(8):888–92.
- [9] Huygen K, Content J, Denis O, Montgomery DL, Yawman AM, Deck RR, et al. Immunogenicity and protective efficacy of a tuberculosis DNA vaccine. *Nat Med* 1996;2(8):893–8.
- [10] Lowrie DB, Silva CL, Colston MJ, Ragno S, Tascon RE. Protection against tuberculosis by a plasmid DNA vaccine. *Vaccine* 1997;15(8):834–8.
- [11] Reed SG, Alderson MR, Dalemans W, Lobet Y, Skeiky YAW. Prospects for a better vaccine against tuberculosis. *Tuberculosis (Edinburgh)* 2003;83:213–9.
- [12] Burke RL, Pacht C, Quiroga M, Rosenberg S, Haigwood N, Nordfang O, et al. The functional domains of coagulation factor VIII:C. *J Biol Chem* 1986;261(27):12574–8.
- [13] Chapman BS, Thayer RM, Vincent KA, Haigwood NL. Effect of intron A from human cytomegalovirus (Towne) immediate-early gene on heterologous expression in mammalian cells. *Nucl Acids Res* 1991;19(14):3979–86.
- [14] Leitner WW, Seguin MC, Ballou WR, Seitz JP, Schultz AM, Sheehy MJ, et al. Immune responses induced by intramuscular or gene gun injection of protective deoxyribonucleic acid vaccines that express the circumsporozoite protein from *Plasmodium berghei* malaria parasites. *J Immunol* 1997;159(12):6112–9.
- [15] Berg DT, Grinnell BW. Signal and propeptide processing of human tissue plasminogen activator: activity of a pro-tPA derivative. *Biochem Biophys Res Commun* 1991;179(3):1289–96.
- [16] Baldwin SL, D'Souza CD, Orme IM, Liu MA, Huygen K, Denis O, et al. Immunogenicity and protective efficacy of DNA vaccines encoding secreted and non-secreted forms of *Mycobacterium tuberculosis* Ag85A. *Tuber Lung Dis* 1999;79(4):251–9.
- [17] Kohne C, Johnson A, Tom S, Peers DH, Gehant RL, Hotaling TA, et al. Secretion of glycosylation site mutants can be rescued by the signal/pro sequence of tissue plasminogen activator. *J Cell Biochem* 1999;75(3):446–61.
- [18] Baldwin SL, D'Souza CD, Roberts AD, Kelly BP, Frank AA, Liu MA, et al. Evaluation of new vaccines in the mouse and guinea pig model of tuberculosis. *Infect Immun* 1998;66(6):2951–9.
- [19] Horwitz MA, Harth G. A new vaccine against tuberculosis affords greater survival after challenge than the current vaccine in the guinea pig model of pulmonary tuberculosis. *Infect Immun* 2003;71(4):1672–9.
- [20] Lasco TM, Cassone L, Kamohara H, Yoshimura T, McMurray DN. Evaluating the role of tumor necrosis factor-alpha in experimental pulmonary tuberculosis in the guinea pig. *Tuberculosis (Edinburgh)* 2005;85(4):245–58.
- [21] Olsen AW, Williams A, Okkels LM, Hatch G, Andersen P. Protective effect of a tuberculosis subunit vaccine based on a fusion of Antigen85B and ESAT-6 in the aerosol guinea pig model. *Infect Immun* 2004;72(10):6148–50.
- [22] Orme IM. Current progress in tuberculosis vaccine development. *Vaccine* 2005;23(17):2105–8.
- [23] Orme IM, McMurray DN, Belisle JT. Tuberculosis vaccine development: recent progress. *Trends Microbiol* 2001;9(3):115–8.
- [24] Williams A, Hatch GJ, Clark SO, Gooch KE, Hatch KA, Hall GA, et al. Evaluation of vaccines in the EU TB vaccine cluster using a guinea pig aerosol infection model of tuberculosis. *Tuberculosis (Edinburgh)* 2005;85(1–2):29–38.
- [25] McMurray DN. Guinea pig model of tuberculosis. In: Bloom BR, editor. *Tuberculosis: pathogenesis, protection and control*. Washington, DC: ASM Press; 1994. p. 135–47.
- [26] Valdivia RH, Falkow S. Fluorescence-based isolation of bacterial genes expressed within host cells. *Science* 1997;277(5334):2007–11.
- [27] Bacon JB, James BW, Wernisch L, Williams A, Morley KA, Hatch GJ, et al. The influence of reduced oxygen availability on pathogenicity and gene expression in *Mycobacterium tuberculosis*. *Tuberculosis (Edinburgh)* 2004;84(3–4):205–17.
- [28] Hampshire T, Soneji S, Bacon J, James BW, Hinds J, Laing K, et al. Stationary phase gene expression of *Mycobacterium tuberculosis* following a progressive nutrient depletion: a model for persistent organisms? *Tuberculosis (Edinburgh)* 2004;84(3–4):228–38.
- [29] Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, et al. *Short protocols in molecular microbiology*. Chichester, USA: Wiley; 1992.
- [30] Shuttleworth H, Ambrose E, Griffiths K, MacDonald-Fyall J, Minton N, Rawkins A, et al. Identification of novel TB vaccine candidates by differential fluorescence induction. *Tuberculosis (Edinburgh)* 2002;82(4–5):209.
- [31] <http://www.chem.agilent.com/scripts/pds.asp?lpage=27881>.

- [32] Nielsen H, Engelbrecht J, Brunak S, von Heijne G. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng* 1997;10(1):1–6.
- [33] Williams A, Davies A, Marsh PD, Chambers MA, Hewinson RG. Comparison of the protective efficacy of Bacille Calmette-Guerin vaccination against aerosol challenge with *Mycobacterium tuberculosis* and *Mycobacterium bovis*. *Clin Infect Dis* 2000;30(Suppl 3):S299–301.
- [34] Karlin S, Mrazek J. Predicted highly expressed genes of diverse prokaryotic genomes. *J Bact* 2000;182(18):5238–50.
- [35] Mustafa AS, Oftung F, Amoudy HA, Madi NM, Abal AT, Shaban F, et al. Multiple epitopes from the *Mycobacterium tuberculosis* ESAT-6 antigen are recognized by antigen-specific human T cell lines. *Clin Infect Dis* 2000;30(Suppl 3):S201–5.
- [36] Sassetti CM, Boyd DH, Rubin EJ. Genes required for mycobacterial growth defined by high density mutagenesis. *Mol Microbiol* 2003;48(1):77–84.
- [37] Manganello R, Voskuil MI, Schoolnik GK, Dubnau E, Gomez M, Smith I. Role of the extracytoplasmic-function σ factor σ^H in *Mycobacterium tuberculosis* global gene expression. *Mol Microbiol* 2002;45(2):365–74.
- [38] Schnappinger D, Ehrt S, Voskuil MI, Liu Y, Mangan JA, Monahan IM, et al. Transcriptome adaptation of *Mycobacterium tuberculosis* within macrophages: insights into the phagosomal environment. *J Exp Med* 2003;198(5):693–704.
- [39] Howard RJ. Vaccination against malaria: recent advances and the problems of antigenic diversity and other parasite evasion mechanisms. *Int J Parasitol* 1987;17(1):17–29.
- [40] Okkels LM, Brock I, Follmann F, Agger EM, Arend SM, Ottenhoff TM, et al. PPE protein (Rv3873) from DNA segment RD1 of *Mycobacterium tuberculosis*: strong recognition of both specific T-cell epitopes and epitopes conserved within the PPE family. *Infect Immun* 2003;71(11):6116–23.
- [41] Delogu G, Brennan MJ. Comparative immune response to PE and PE_{PGRS} antigens of *Mycobacterium tuberculosis*. *Infect Immun* 2001;69(9):5606–11.
- [42] Eswaramoorthy S, Kumaran D, Swaminathan S. A novel mechanism for *Clostridium botulinum* neurotoxin inhibition. *Biochemistry* 2002;41(31):9795–802.
- [43] Kochi SK, Schiavo G, Mock M, Montecucco C. Zinc content of the *Bacillus anthracis* lethal factor. *FEMS Microbiol Lett* 1994;124(3):343–8.
- [44] Kooi C, Corbett CR, Sokol PA. Functional analysis of the *Burkholderia cenocepacia* ZmpA metalloprotease. *J Bacteriol* 2005;187(13):4421–9.
- [45] Shoop WL, Xiong Y, Wiltsie J, Woods A, Guo J, Pivnichny JV, et al. Anthrax lethal factor inhibition. *Proc Natl Acad Sci* 2005;102(22):7958–63.
- [46] Derrick SC, Repique C, Snoy P, Yang AL, Morris S. Immunization with a DNA vaccine cocktail protects mice lacking CD4 cells against an aerogenic infection with *Mycobacterium tuberculosis*. *Infect Immun* 2004;72(3):1685–92.
- [47] Lima KM, dos Santos SA, Santos RR, Brandao IT, Rodrigues JM, Silva CL. Efficacy of DNA-hsp65 vaccination for tuberculosis varies with method of DNA introduction *in vivo*. *Vaccine* 2003;22(1):49–56.
- [48] Wang R, Doolan DL, Le TP, Hedstrom, Coonan KM, Charoenvit Y, et al. Induction of antigen-specific cytotoxic T lymphocytes in humans by a malaria DNA vaccine. *Science* 1998;282(5388):476–80.
- [49] Brandt L, Skeiky YAW, Alderson MR, Lobet Y, Dalemans W, Turner OC, et al. The protective effect of the *Mycobacterium bovis* BCG vaccine is increased by co administration with the *Mycobacterium tuberculosis* 72-kDa fusion polypeptide Mtb72F in *M. tuberculosis*-infected guinea pigs. *Infect Immun* 2004;72(11):6622–32.
- [50] Skeiky YAW, Alderson MA, Owendale PJ, Guderian JA, Brandt L, Dillon DC, et al. Differential immune responses and protective efficacy induced by components of a tuberculosis polyprotein vaccine, Mtb72F, delivered as naked DNA or recombinant protein. *J Immunol* 2004;172(12):7618–28.
- [51] Sugawara I, Yamada H, Udagawa T, Huygen K. Vaccination of guinea pigs with DNA encoding Ag85A by gene gun bombardment. *Tuberculosis (Edinburgh)* 2003;83:331–7.
- [52] Mollenkopf HJ, Grode L, Mattow J, Stein M, Mann P, Knapp B, et al. Application of Mycobacterial proteomics to vaccine design: Improved protection by *Mycobacterium bovis* BCG prime-Rv3407 DNA boost vaccination against tuberculosis. *Infect Immun* 2004;72(11):6471–9.
- [53] Giri M, Ugen KE, Weiner DB. DNA vaccines against human immunodeficiency virus type 1 in the past decade. *Clin Microbiol Rev* 2004;17(2):370–89.
- [54] Moore AC, Hill AVS. Progress in DNA-based heterologous prime-boost immunization strategies for malaria. *Immunol Rev* 2004;199:126–43.