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Delegatura de propiedad Industrial

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SOLICITUD

PATENTE DE INVENCIÓN

21. **EXPEDIENTE No.** 07-3868

54. **TÍTULO** Tratamiento de Infecciones Bacterianas

51. **CLASIFICACIÓN INTERNACIONAL** C07K 16/12, A61K 39/3

71. **SOLICITANTE** Newtec Pharma PLC

DOMICILIO Manchester, Reino Unido

74. **APODERADO** Dilia María Rodríguez D' Aleman

22. **BOGOTÁ, D. C.,** Enero 16/07.

(FORMA P-10)

PETITORIO

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SUPERINTENDENCIA

FORMULARIO ÚNICO DE SOLICITUD DE PATENTE PCT ENTRADA EN FASE NACIONAL

1) SOLICITUD DE:

[X] Patente de invención   [ ] Patente de Modelo de Utilidad

[ ] Capitulo I.   [X] Capitulo II.

2) SOLICITANTE (71)	**Nombre: NEUTEC PHARMA PLC** **Dirección: Central Manchester & Manchester Children´s University Hospital NHS Trust Oxford Road, Manchester M13 9WL Reino Unido** **Nacionalidad o domicilio: Manchester, Reino Unido** **Lugar de constitución: Reino Unido**	**IDENTIFICACIÓN** C.C. [ ] NIT: [ ] C.E. [ ] OTRO [ ] Cual _____ NÚMERO _____
	Teléfono: Fax: **E. Mail:**	
3) REPRESENTANTE O APODERADO	Nombre: DILIA MARÍA RODRÍGUEZ D´ALEMAN Dirección: Carrera 11 N°. 86-53 Piso 6 Bogotá D.C. Teléfono: 6181088 Fax: 6350824 E. Mail: info@clarkemodet.com.co	**IDENTIFICACIÓN** C.C. [X] NIT: [ ] C.E. [ ] OTRO [ ] NÚMERO: 41.654.882 TP: 20824 CSJ
4) INVENTOR (ES) (72)	**Nombre: 1. BURNIE, James Peter; 2. MATTHEWS, Ruth, Christine; 3. CARTER, Tracey** **Dirección: Todos los anteriores, Central Manchester & Manchester Children´s University Hospital NHS Trust Oxford Road, Manchester M13 9WL Reino Unido** **Nacionalidad o Domicilio: Todos los anteriores Manchester, Reino Unido**	

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Instrucciones para el diligenciamiento del presente formulario. Ver reverso de esta pagina

1

97

10) ANEXOS

- [x] Comprobante de pago de la tasa de presentación de la solicitud
- [ ] Comprobante de pago por reivindicación de prioridad
- [ ] Documento que acredita la existencia y representación legal cuando el solicitante sea persona *Jurídica*
- [ ] Poderes, si fuera el caso
- [ ] Documento de cesión del inventor al solicitante o a su causante
- [ ] Declaraciones
- [x] Copia de la solicitud Internacional. (Resumen, descripción, reivindicación, Dibujos).
- [x] Traducción de la solicitud internacional al castellano. (Resumen, descripción, reivindicación, Dibujos)
- [ ] Copia del examen preliminar efectuado por la IPEA
- [ ] De ser el caso, copia del contrato de acceso
- [ ] De ser el caso, documento que acredite la licencia o autorización de uso de conocimientos tradicionales de las comunidades indígenas
- [ ] De ser el caso, certificado de depósito del material biológico
- [ ] Arte final 12 x 12 y 6 x 6 cm
- [ ] De ser el caso, información sobre otras solicitudes de patente o títulos obtenidos en el extranjero por el mismo titular o su causante, relacionados parcial o totalmente con la invención de esta solicitud.
- [x] Otros:

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11) FIGURA CARACTERÍSTICA:

12) Solicitud concesión de la patente,

NOMBRE: DILIA RODRÍGUEZ D´ALEMAN

FIRMA:

C.C. 41.654.882 de Bogota **TP** 20824 CSJ

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[illegible]RKE MODET Y CO. COLOMB	CONSIGNACION	BANCO POPULAR	050-00110-6	55053479	28/12/2006	5,705,000.00

C O N C E P T O

CANT.	RENTISTICO		CONCEPTO	TOTAL CONCEPTO
1	50005-01-01	SOLICITUDES	1 TRAMITES DE SOL. DE PATENTE DE IN	463,000.00
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SON: CUATROCIENTOS SESENTA Y TRES MIL PESOS

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a

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CANT.	RENTISTICO		CONCEPTO		TOTAL CONCEPTO
1	50005-01-09	OTROS SERVICIOS DE PROPIEDAD I	998	VALOR COMPLEMENTO TARIFA A¥O ANTR	19,000.00
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(54) Title: TREATMENT OF BACTERIAL INFECTIONS

(57) Abstract: The present invention is concerned with compounds, medicaments and treatments for Clostridium difficile infection, together with novel isolated antibodies and their use in same. The present invention is also concerned with the treatment and prophylaxis of Escherichia and Enterococcus infection and provides medicaments and treatments for same.

Treatment of Bacterial Infections

The present invention is concerned with compounds, medicaments and treatments for *Clostridium difficile* infection, together with novel isolated antibodies and their use in same. The present invention is also concerned with the prophylaxis and treatment of *E.faecium* and *E.faecalis* infection and provides medicaments and treatments for same.

C. difficile is a gram-positive anaerobic bacterium, and is deemed a significant human pathogen causing a spectrum of diseases ranging from mild diarrhoea to fulminant pseudomembranous colitis (PMC) - collectively referred to as *C. difficile* antibiotic-associated diarrhoea (CDAD). CDAD is a common, iatrogenic, nosocomial disease associated with substantial morbidity and mortality, especially in the elderly. Two factors have been assigned main roles in the pathogenesis of CDAD - the suppression of the resident intestinal flora by the administration of antibiotics, and the production by the bacterium of two high molecular weight toxins, exotoxin A and exotoxin B.

The bacterium is endemic in hospitals, and studies have shown that approximately one third of patients receiving antibiotic treatment in acute-care medical wards were colonised by *C. difficile* while in hospital (Kyne, L., *et al.*, 2002, Clin. Infect. Dis. 34(3), pp346-53, PMID: 11774082). Of these patients, over half went on to develop CDAD while the remainder were symptomless carriers. CDAD is a major factor in extension of patient hospital stay times, and estimates suggest that the cost of this disease in the US exceeds $1.1 billion per year (Kyne, L., *et al.*, Supra). Patients suffering from CDAD respond well to a treatment which includes a discontinuation of the inciting antibiotic and treatment with either of the antibiotics metronidazole and vancomycin. The response rate to initial antibiotic therapy can be high (up to 95%), but up to 20% of patients can relapse within one or two weeks of a first treatment, with the risk of relapse compounding with each

relapse episode. Relapse can typically be treated with antibiotics, indicating that it is due to infection with different *C.difficile* strains. Additionally, there is evidence that *C. difficile* is becoming resistant to metronidazole and partially resistant to vancomycin, demonstrating the need for new alternatives in the treatment of CDAD.

Exotoxins A and B which are produced by pathogenic strains of the bacterium are cytotoxic, enterotoxic and proinflammatory, and are considered to be the main virulence factors of this non-invasive microorganism. However, not all infections with toxigenic strains result in disease, prompting the search for additional virulence factors. Bacterial surface expressed antigens represent candidate virulence factors, and are also considered important since such proteins likely mediate the essential functions such as adhesion to the epithelial layer of the gut in the first step of colonization or interaction with mediators of local immunity. In common with many other bacteria, *C. difficile* expresses a crystalline or paracrystalline surface layer (S-layer) on the outer cell surface. Such S-layers comprise proteins or glycoproteins forming a regularly arranged lattice on the external surface of the bacterium, and have previously been shown to be essential for the virulence of pathogens such as *Aeromanas salmonicida* and *Campylobacter fetus*. In contrast to most bacteria which comprise one S-layer, *C. difficile* is known to comprise two superimposed paracrystalline S-layers, each composed of a glycoprotein subunit which varies slightly in apparent molecular weight among different *C. difficile* strains. Most strains of *C. difficile* express two major S-layer proteins (SLPs), one of 32-38 kDa (low-MW SLP) and a second of 42-48 kDa (high-MW SLP). The low-MW SLP appears to be immunodominant and is the antigen most commonly recognised by patients suffering from CDAD, and is the only antigen recognised in EDTA extracts of bacteria by antisera raised in rabbits against whole *C. difficile* cells (Calabi, E. *et al.*, 2001, Mol. Microbiol., 40(5) p1187-99, PMID: 11401722).

During the course of microbial infection various adaptive strategies are employed by the immune system. One such strategy, and arguably the most important, is the production of an antibody response. Antibodies capable of binding antigens displayed by the infectious agent are produced and bind to and allow killing of the microorganism through complement activation, recruitment of macrophage and through direct interaction with the microbe itself. The therapeutic efficacy of antibodies capable of binding a given antigen varies and this is reflected in the fact that antibody production by the immune system matures during the course of an infection and becomes focussed in the case of a patient successfully fighting off an infection.

The antibody response is elicited by the B cell repertoire where individual B cells each produce structurally diverse antibody molecules. The actual size of this B cell/antibody repertoire is unknown, but it is estimated that the random clonal frequency of reactivity for a given antigen may be as high as 1 in 100,000 in cultured B cells (Nobrega, A., et al., Eur J Immunol. 1998 Apr;28(4):1204-15; PMID: 9565360). During the course of infection, antibodies capable of binding the pathogen are selected for by changes in the B cell population resulting in key antibodies being produced in large numbers. The mechanisms for these changes include clonal expansion, isotype switching, and somatic mutation of immunoglobulin variable regions. B cells responsible for generating antibodies which are able to bind a pathogen multiply, thus skewing the B cell repertoire and changing the proportions of B cells.

Antibody specific against the cell-binding domain of the *C.difficile* exotoxin A is found to be protective in a mouse model, and patients colonized with *C.difficile* but asymptotic for it are found to have an elevated anti-exotoxin A IgG titer. Infected patients developing such elevated serum anti-exotoxin A IgG titers in response to colonization are 48 times less likely to suffer from CDAD than patients who did not develop such an elevated titer. Therefore vaccination with *C.difficile* exotoxin A is suggested as a therapeutic strategy,

as is the use of anti-*C.difficile* exotoxin A antibodies (Giannasca PJ and Warny M., Vaccine, 2004 Feb 17;22(7): 848-56; PMID: 15040937).

Non-antibiotic based therapeutic regimes for the treatment/prevention of *C. difficile* infection are based upon vaccination and passive immunization. Vaccination treatment comprises administering to a patient either a nucleic acid sequence encoding an immunogenic fragment of the *C. difficile* surface layer protein or a variant or homologue thereof, or an equivalent polypeptide fragment (as disclosed in WO 02/062379). Passive immunotherapy is typically achieved by administering to a patient a monoclonal antibody specific to an immunogen produced by a pathogen. In general, passive immunotherapy is particularly effective in treating immunocompromised patients who are unable to respond to vaccination, and to patients who need immediate therapy and cannot wait for vaccination to take effect. In the case of a *C. difficile* infection, passive immunization relies on the administration to a patient of toxin-neutralizing polyclonal immune globulin, (as disclosed in WO 99/20304), or antibodies raised against the whole bacterium and the toxins (as disclosed in WO 96/07430).

Therefore the effective treatment of *C.difficile* infection is highly important, and the prior art teaches that the target for such therapy should be vaccination or passive immunotherapy targeted against the *C.difficile* exotoxin A, against the surface layer protein, or as a polyclonal serum specific against unidentified bacterial toxins.

However, the present inventor has identified a specific target for therapy. The experiments below show that patients infected with *C.difficile* produce an elevated titre of antibodies specific against acetyl-CoA acetyltransferase (thiolase), and that anti-acetyl-CoA acetyltransferase (thiolase) antibody *per se* is useful and effective in treating *C.difficile* infection. Furthermore, anti-acetyl-CoA acetyltransferase antibody, particularly a synthetic antibody constructed from the most dominant CDR sequences from *C.difficile* infected

patients, when used together with the antibiotics vancomycin or gentamicin, results in synergistic therapy of *C.difficile* infection.

Thus according to a first aspect of the present invention there is provided the use of an inhibitor of acetyl-CoA acetyltransferase in the manufacture of a medicament for the treatment or prophylaxis of infection by *Clostridium difficile*.

As used herein, the term "treatment" is intended to have a broad meaning unless explicitly stated otherwise. Thus by "treatment" or "therapy" is meant any treatment which is designed to cure, alleviate, remove or lessen the symptoms of, or prevent or reduce the possibility of contracting disorders or malfunctions of the human or animal body. Thus by the term "treatment" is meant both treatment of disease conditions, as well as their prophylaxis.

In particular, the acetyl-CoA acetyltransferase may be from *Clostridium difficile*, and it may have the sequence of SEQ ID NO: 43.

A wide range of inhibitors may be used which are specific against the acetyl-CoA acetyltransferase. In particular, the acetyl-CoA acetyltransferase may form a specific binding pair (sbp) with the inhibitor, the acetyl-CoA acetyltransferase being the first member of the pair, and the inhibitor being the second member.

Herein, a "Member of a Specific Binding Pair" is one of two different molecules, having an area on the surface or in a cavity which specifically binds to and is thereby defined as complementary with a particular spatial and polar organization of the other molecule. The members of the specific binding pair are referred to as ligand and receptor (antiligand), sbp member and sbp partner, sbp members or the like. These are usually members of an immunological pair such as antigen-antibody, although the term does have a broader

meaning encompassing other specific binding pairs, such as RNA-protein, DNA-protein, and the like.

Thus the inhibitor may be an antibody or an antigen binding fragment thereof, and it may be specific against an epitope displayed by the peptide of SEQ ID NO: 47

As detailed below in the Experiments section, the epitope displayed by the peptide having the sequence of SEQ ID NO: 47 has been identified as being conserved between various antigens and is therefore identified as being the peptide displaying the conserved epitope shared by those antigens.

The term "antibody" in its various grammatical forms is used herein to refer to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e. molecules that contain an antibody combining site or paratope. Such molecules are also referred to as "antigen binding fragments" of immunoglobulin molecules.

Illustrative antibody molecules are intact immunoglobulin molecules, substantially intact immunoglobulin molecules and those portions of an immunoglobulin molecule that contain the paratope, including those portions known in the art as Fab, Fab', F(ab')2, scFv and F(v). Antibodies, their production and use are well known in the art (e.g. Harlow, E. and Lane, D., "Using Antibodies: A Laboratory Manual", Cold Spring Harbor Laboratory Press, New York, 1998).

As detailed in the experiments below, the variable heavy (VH) and variable light (VL) chains of antibodies produced by patients infected with *C.difficile* have been cloned and sequenced and their complementarity determining regions (CDRs) identified. This has shown that there are highly immunodominant VH chain CDRs 1-3 (CDR-H1, CDR-H2

and CDR-H3), and highly immunodominant VL chain CDRs 1-3 (CDR-L1, CDR-L2 and CDR-L3).

Thus, the antibody or antigen binding fragment thereof may have VH chain complementarity determining regions (CDRs) 1-3 having the sequences of SEQ ID NOs: 2-4 respectively. In particular, the antibody or antigen binding fragment thereof may have a VH chain having the sequence of SEQ ID NO: 1.

Similarly, the antibody or antigen binding fragment thereof may have VL chain complementarity determining regions (CDRs) 1-3 having the sequences of SEQ ID NOs: 17-19. In particular, the antibody or antigen binding fragment thereof may have a VL chain having the sequence of SEQ ID NO: 16.

A synthetic antibody having the above sequences has been created and used, and thus the antibody may have the sequence of SEQ ID NO: 41. The antibody detailed below having SEQ ID NO: 41 also comprises an N-terminal S-Tag and a C-terminal His-Tag. The tags are useful for experimental purposes but are unnecessary for the therapeutic antibody, although they (or other tags) may be considered useful. Thus the antibody may e.g. additionally comprise a His-Tag, for example a C-terminal His-Tag.

The experiments below have shown that not only is an inhibitor of acetyl-CoA acetyltransferase effective on its own in effecting treatment of *Clostridium difficile* infection, but it is also useful when used in conjunction with existing antibiotics, particularly gentamicin and vancomycin, and that such use can provide synergistic results. Preliminary experimental results also show that synergy is achieved with metronidazole.

Thus, the medicament may additionally comprise at least one of the group of antibiotics consisting: gentamicin, vancomycin and metronidazole.

Naturally, medical formulations of the present invention may comprise a pharmaceutically acceptable carrier, diluent or excipient (Remington's Pharmaceutical Sciences and US Pharmacopoeia, 1984, Mack Publishing Company, Easton, PA, USA; United States Pharmacopoeia, ISBN: 1889788031).

As detailed above, an inhibitor of acetyl-CoA acetyltransferase effective on its own in effecting treatment of *Clostridium difficile* infection. Thus also provided according to the present invention is isolated and/or purified antibody comprising at least one of the group consisting:

(i) VH chain complementarity determining regions (CDRs) 1-3 having the sequences of SEQ ID NOs: 2-4 respectively; and

(ii) VL chain complementarity determining regions (CDRs) 1-3 having the sequences of SEQ ID NOs: 17-19.

As above, the VH chain may have the sequence of SEQ ID NO: 1. The VL chain may have the sequence of SEQ ID NO: 16. The antibody may have the sequence of SEQ ID NO: 41.

Also provided is a nucleic acid molecule coding for such an antibody. The nucleic acid molecule may be isolated and/or purified. In particular, the nucleic acid molecule may have the sequence of SEQ ID NO: 46.

Also provided according to the present invention is the use of an inhibitor of acetyl-CoA acetyltransferase and vancomycin in the manufacture of a medicament for the prophylaxis or treatment of infection by *Enterococcus faecium*, or *Enterococcus faecalis*, particularly vancomycin resistant *Enterococcus faecium*.

The experiments below show that not only does the use of an inhibitor of acetyl-CoA acetyltransferase and vancomycin result in synergistic therapy of *C.difficile* infection, but

it also results in synergistic therapy of *E.faecium* infection, particularly vancomycin resistant *E.faecium* infection. Preliminary experiments have also shown that synergy is also observed in the treatment of *E.faecalis* infection.

The acetyl-CoA acetyltransferase may be from *Clostridium difficile*.

The inhibitor may be an antibody or an antigen binding fragment thereof, and it may be specific against an epitope displayed by the peptide having the sequence of SEQ ID NO: 47.

As with other aspects of the present invention, the antibody or antigen binding fragment thereof may have VH chain complementarity determining regions (CDRs) 1-3 having the sequences of SEQ ID NOs: 2-4 respectively. The antibody or antigen binding fragment thereof VH chain may have the sequence of SEQ ID NO: 1.

The antibody or antigen binding fragment thereof may have VL chain complementarity determining regions (CDRs) 1-3 having the sequences of SEQ ID NOs: 17-19. The antibody or antigen binding fragment thereof VL chain may have the sequence of SEQ ID NO: 16.

The antibody or antigen binding fragment thereof may have the sequence of SEQ ID NO: 41.

Where the present invention is concerned with the provision of medicaments containing two or more active ingredients (for example antibody and an antibiotic), the present invention also provides combined preparations comprising such active ingredients. For example, two-pack preparations may be provided.

Thus also provided according to the present invention is a combined preparation for the treatment of infection by *Clostridium difficile*, comprising:

(i) an inhibitor of acetyl-CoA acetyltransferase; and

(ii) at least one of the group of antibiotics consisting: gentamicin, vancomycin, and metronidazole.

Also provided is a combined preparation for the treatment of infection by *Enterococcus faecium* or *Enterococcus faecalis*, comprising:

(i) an inhibitor of acetyl-CoA acetyltransferase; and

(ii) vancomycin.

The *Enterococcus faecium* or *Enterococcus faecalis* may be vancomycin resistant.

The present invention also extends to methods of treatment of patients. Thus also provided according to the present invention is a method of treatment of infection by *Clostridium difficile*, comprising administering a therapeutically effective quantity of an inhibitor of acetyl-CoA acetyltransferase to a patient in need of same. The method may additionally comprise administering a therapeutically effective quantity of at least one of the group of antibiotics consisting: gentamicin, vancomycin and metronidazole.

Also provided according to the present invention is a method of treatment of infection by *Enterococcus faecium* or *Enterococcus faecalis*, comprising administering a therapeutically effective quantity of:

(i) an inhibitor of acetyl-CoA acetyltransferase; and

(ii) vancomycin

to a patient in need of same.

The *Enterococcus faecium* may be vancomycin resistant.

The invention will be further apparent from the following description, which show, by way of example only, forms of treatment of *C.difficile* and *E.faecium* infection.

Experiments

In the experiments below, a synthetic antibody has been constructed using the most predominant VH and VL antibody sequences from patients infected with *C.difficile*. Using this synthetic antibody, an antigen was isolated and purified and electrospray mass spectrometry used to determine a putative partial sequence for the isolated protein. Searching of *C.difficile* genomic sequences for matches identified a possible candidate matching sequence. A comparison of the candidate *C.difficile* sequence identified a number of homologous proteins which are classified as acetyl-CoA acetyltransferase (thiolase) enzymes and confirms it as a member of that family. The closest match is NP_349376 from *Clostridium acetobutylicum*, which showed a 68% homology over 391 amino acids. Cloning, expression and purification of the candidate protein gave a protein which bound strongly with the synthetic antibody. Further experiments have shown that the antibody specific against the *C.difficile* acetyl-CoA acetyltransferase (thiolase) shows synergy with vancomycin and gentamicin in treating *C.difficile* infections. Preliminary experiments also show that synergy can also be achieved with metronidazole.

Experiments also show synergy between the synthetic antibody and vancomycin in the treatment of vancomycin resistant *E.faecium*. Preliminary experiments (not shown) also indicate synergy between the synthetic antibody and vancomycin in the treatment of *E.faecalis* infection.

Unless stated otherwise, all procedures were performed using standard protocols and following manufacturer's instructions where applicable. Standard protocols for various techniques including PCR, molecular cloning, manipulation and sequencing, the manufacture of antibodies, epitope mapping and mimotope design, cell culturing and phage display, are described in texts such as McPherson, M.J. *et al.* (1991, PCR: A practical approach, Oxford University Press, Oxford), Sambrook, J. *et al.* (1989, Molecular

cloning: a laboratory manual, Cold Spring Harbour Laboratory, New York), Huynh and Davies (1985, "DNA Cloning Vol I - A Practical Approach", IRL Press, Oxford, Ed. D.M. Glover), Sanger, F. *et al.* (1977, PNAS USA 74(12): 5463-5467), Harlow, E. and Lane, D. ("Using Antibodies: A Laboratory Manual", Cold Spring Harbor Laboratory Press, New York, 1998), Jung, G. and Beck-Sickinger, A.G. (1992, Angew. Chem. Int. Ed. Eng., 31: 367-486), Harris, M.A. and Rae, I.F. ("General Techniques of Cell Culture", 1997, Cambridge University Press, ISBN 0521 573645), "Phage Display of Peptides and Proteins: A Laboratory Manual" (Eds. Kay, B.K., Winter, J., and McCafferty, J., Academic Press Inc., 1996, ISBN 0-12-402380-0).

Reagents and equipment useful in, amongst others, the methods detailed herein are available from the likes of Amersham (www.amersham.co.uk), Boehringer Mannheim (www.boehringer-ingelheim.com), Clontech (www.clontech.com), Genosys (www.genosys.com), Millipore (www.millipore.com), Novagen (www.novagen.com), Perkin Elmer (www.perkinelmer.com), Pharmacia (www.pharmacia.com), Promega (www.promega.com), Qiagen (www.qiagen.com), Sigma (www.sigma-aldrich.com) and Stratagene (www.stratagene.com).

The contents of each of the references discussed herein, including the references cited therein, are herein incorporated by reference in their entirety.

Where "PMID:" reference numbers are given for publications, these are the PubMed identification numbers allocated to them by the US National Library of Medicine, from which full bibliographic information and abstract for each publication is available at www.ncbi.nlm.nih.gov. This can also provide direct access to electronic copies of the complete publications, particularly in the case of e.g. PNAS, JBC and MBC publications.

Sequence homology is as determined using the BLAST2 program (Tatusova TA *et al.*, FEMS Microbiol Lett. 1999 May 15;174(2):247-50; PMID: 10339815) at the National Center for Biotechnology Information, USA (www.ncbi.nlm.nih.gov) with default parameters.

Identifying dominant VH and VL antibody sequences from patients infected with *C.difficile*

In order to identify antibody sequences from patients with *C.difficile* infections and determine the dominant antibody sequences, particularly CDR sequences, the methods detailed in WO 03/052416 were used. B cells producing antibodies were identified, sequenced and analysed, using the following basic steps (see WO 03/052416):

(1) Isolation of VH and/or VL coding regions from circulatory B cells of human patients.

(2) Determining the Nucleotide sequence of VH and/or VL repertoires.

(3) Determination of VH and/or VL primary amino acid sequences.

(4) Extraction of CDR regions *in silico* - incorporation into the database

(5) Detection of dominant CDR & framework regions in VH and/or VL repertoire.

(6) Construction and Production of therapeutic recombinant antibodies from dominant antibody sequences.

Fragments of antibodies were sequenced from four infected patients (D01, D02, D03 and D04).

Heavy Chain (CDH1)

CDH1 was the most commonly occurring VH chain from the *C.difficile* infected patients. It was identified from analysis of the CDR3 sequences from patient antibody VH chains. 226/1011 (22.4%) of cloned antibodies from *C.difficile* infected patients had the same

CDR3 sequence (CDR-H3, below). CDH1 was found to be present in three quarters of patients. Its CDR3 sequence appeared in 184/318 (57.9%) of the clones from patient D01; in D03 it appeared in 40/291 (13.7%) of the clones; and in D04 it appeared in 2/252 (0.8%) of the clones. The most common full VH sequence is SEQ ID NO: 1.

Within this VH chain sequence, the CDR (complementarity determining region) sequences are as follows:

CDR-H1	SEQ ID NO: 2
CDR-H2	SEQ ID NO: 3
CDR-H3	SEQ ID NO: 4

Homology searches have identified several other CDR3 sequences with greater than 70% homology to the CDR-H3 sequence (SEQ ID NO: 4), all from *C.difficile* patients. The CDR3 sequences are SEQ ID NOs: 5-15.

<u>Light Chain (CDL1)</u>

The light chain for H1L1 was derived from a clone with the most common CDR3 sequence from *C.difficile* infected patients. CDL1 was present in patient D01 at a frequency of 84/251 (33.5%). The most common VL sequence containing this CDR3 sequence was SEQ ID NO: 16.

Within this VL chain sequence, the CDR (complementarity determining region) sequences are as follows:

CDR-L1	SEQ ID NO: 17
CDR-L2	SEQ ID NO: 18
CDR-L3	SEQ ID NO: 19

Homology searches have identified several other CDR3 sequences with greater than 70% homology to the CDR-L3 sequence, all from *C.difficile* patients. The CDR3 sequences are SEQ ID NOs: 20-40.

Construction and Cloning of Antibody H1L1

The synthetic antibody H1L1 was constructed as follows: CDH1 and CDL1 were separately PCR amplified from the sequencing vector and cloned into cloning vector pGEM-T easy (Promega Corporation) to facilitate DNA sequencing. For this 3 μg PCR product was prepared for restriction using QIAquick PCR purification spin columns (Qiagen, UK) according to the manufacturers instructions. DNA was eluted from the spin column in 40 μL buffer EB. Purified PCR product (2 μL) was mixed with 1 μL pGEM-T easy vector, 6 μL water and 1 μL DNA ligase and the mixture ligated for 1 h at room temperature. Ligations were then transformed into electrocompetent *E. coli* TG1 cells (Stratagene) by electroporation, and plated out onto agar plates containing Ampicillin 100 μg ml^{-1} IPTG (100 μM) and X-gal (0.006 % w/v). Colonies were allowed to grow overnight at 37 °C and then stored at 4 °C. Recombinant colonies are identified as white colonies on this media.

This gave an antibody having the general structure;

S Tag - CDH1 - Linker - CDL1 - His Tag

with the amino acid sequence of SEQ ID NO: 41 with an N-terminal S-Tag and a C-terminal His-Tag.

Identification of the target for Antibody H1L1 - Acetyl-CoA acetyltransferase (Thiolase)

Materials and methods

Sample preparation:

Clostridium difficile cells (NCTC 11204) grown on blood agar plates were suspended in 10 mM of PBS (phosphate buffered saline) and sonicated for 5 x 1 minute on ice. The cell lysate was centrifuged at 13000 rpm for 5 minutes and 300 µl of the supernatant was precipitated in 20 mL 10 % trichloroacetic acid and 20 mM DTT (dithiothreitol) in cold acetone for 45 minutes. The proteins were recovered by centrifugation and the pellet was washed three times with cold acetone containing 20 mM DTT.

2D-gel electrophoresis:

The protein pellet was dissolved in sample rehydration solution (7 M urea, 2 M thiourea, 3% (w/v) CHAPS, 0.002% bromophenol blue in water) and diluted with the same solution for isoelectric focusing. Isoelectric focusing was performed using a Zoom IPGRunner (RTM) System (Invitrogen Ltd, Carlsbad, CA, USA) over a non-linear pH range of 3-10 (7 cm) for a total of 1700 Vh, loading approximately 15 µg of protein on each strip. Prior to the second dimension separation the strips were equilibrated for 15 minutes in equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2 % (w/v) SDS, 0.002% bromophenol blue in water) containing 65 mM DTT and then in the same buffer containing 135 mM iodoacetamide for another 15 minutes.

The second dimension separation was carried out using NuPage 4-12 % Bis-TrisZoom gels and MOPS buffer (Invitrogen Ltd, Carlsbad, CA, USA). The proteins were transblotted onto Invitrolon PVDF membrane (Invitrogen Ltd, Carlsbad, CA, USA) and blocked in 5% skimmed milk in 0.1% Tween20 in 10 mM PBS for 1 hour.

Immunoblotting:

To identify the target of antibody H1L1, the membrane was incubated for 1 hour with purified H1L1 antibody in 5% skimmed milk in 0.1% Tween20 in 10 mM PBS. After washing, an anti-His antibody conjugated with horse radish peroxidase (Santa Cruz

Biotechnologies) was used, diluted at a ratio of 1:500 with 0.1% Tween20 in 10 mM PBS, to detect bound H1L1. After washing with 0.1% Tween20 in 10 mM PBS the blot was developed using ECL (Amersham biosciences, Little Chalfont, UK).

To visualise targets for IgG antibodies in patients infected with *Clostridium difficile*, membranes were incubated for 1 hour with serum diluted 1:20 - 1:50 with 0.1% Tween20 in 10 mM PBS. Anti IgG-antibody conjugated with horse radish peroxidase (for ECL) or alkaline phosphatase (Sigma) where the membrane was developed using SigmaFast (RTM) 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets (Sigma) was used.

Electrospray-mass spectrometry:

Protein bands or spots cut out from the gel were washed in 25 mM ammonium bicarbonate for 10 minutes and dehydrated using 25 mM ammonium bicarbonate:acetonitrile 1:2 for 15 minutes. After repeating the wash with ammonium bicarbonate and the dehydration step, the gel pieces were dried down in a SpeedVac.(RTM). Small volumes of trypsin (10 µg/ml in 25 mM ammonium bicarbonate) were added until gel pieces returned to their original size and a small amount of 25 mM ammonium bicarbonate was added to keep the gel pieces wet during digestion. The samples were digested for 4.5 hours at 37 °C and the peptides extracted for 15 minutes by adding 80 µl acetonitrile. The supernatant was dried in a SpeedVac until all the acetonitrile had evaporated. The sample was desalted using C-18 Zip-Tips (Millipore) and analysed using nano-spray time-of-flight mass spectrometry (Q-TOF, Micromass, Manchester, UK)

Results;

Isolation of target for antibody H1L1 using 2D gels and immunoblotting.

2D gels combined with immunoblotting were used to pinpoint the antigen on the 2D gel. Two blots where compared, one using the serum from the patient whose most abundant antibody was the template for H1L1 and one using the expressed and purified H1L1. Both

reacted to the same protein spot. The same protein was antigenic in four patients who had recovered from diarrhoea due to *C. difficile*.

Isolation of target protein using a column method

A clinical isolate of *C.difficile* (referred to herein as strain CD 14000287, isolated from a *C.difficile* infected patient) was grown anaerobically in brain heart infusion broth supplemented with thioglycolic acid and L-cysteine-HCl for 48 hours at 37 °C. The cell suspension was centrifuged in a Sorval RC-3B centrifuge at 5000 rpm for 20 minutes to pellet the cells and the cell pellet was washed once with PBS and re-centrifuged as above. The washed cell pellet was resuspended in PBS and stored at -20 °C until further use. A 2 ml aliquot of the frozen cell suspension was thawed and centrifuged at 14000 rpm for 5 minutes on a benchtop microfuge to pellet the cells, and the pelleted cells were resuspended in 4 ml of 6 M guanidine-HCl pH 8.0. The guanidine extracted protein was clarified by centrifugation at 45000 rpm on a Beckman L8-70M ultracentrifuge for 1 hour at 20 °C. The supernatant was dialysed at room temperature against three times 2 litres of 20 mM sodium carbonate buffer pH 9.5 to exchange the extracted protein into a compatible buffer for the AminoLink (supplied by Perbio) column. 2 ml of an H1L1 antibody solution (~2 mg/ml) in 20 mM sodium carbonate pH 9.5 was covalently coupled to the AminoLink resin equilibrated with 20 mM sodium carbonate pH 9.5 in a prepacked 2 ml column following the manufacturer's instructions (Perbio). The *C.difficile* antigen containing extract (1.5 ml) was then applied to the covalently coupled H1L1 column and incubated with the resin for 1 hour at room temperature. Unbound material was washed through the column with 12 ml of 20 mM sodium carbonate buffer pH 9.5 and the bound antigen eluted by applying 8 ml of 0.1 M glycine buffer pH 2,5-3.0. The eluted protein was neutralised with 1 M Tris pH 9.0 prior to concentration on an Amicon 15 ml concentrator adaptor to ~200 µl. This concentrated protein material was then analysed by 10 % (w/v) SDS-PAGE and visualised by Coomassie Blue staining. The H1L1 interacting protein band was then identified by mass spectrometry.

Identification of isolated protein using mass spectrometry

The protein band or spot was cut out of the gel and digested at 37 °C with trypsin. The samples were analysed using nano electrospray time-of-flight mass spectrometry, which gave the sequence of SEQ ID NO: 42.

The same peptide was found in the samples from the 2D gel experiments as from the protein isolated using the column based method.

Searching the *Clostridium difficile* genome using BLAST search at the Sanger Institute (www.sanger.ac.uk/projects/C_difficile/) gives SEQ ID NO: 43 as a match.

Theoretical pI: 5.74 Molecular weight: 43430.30 Da which correlates well with the location of the protein on the 2D gel where the molecular weight was approximately 44 kD and pI 5.5-6.

Using the protein sequence of SEQ ID NO: 43 in a protein-protein NCBI BLAST search at www.ncbi.nlm.nih.gov/BLAST/ using default settings reveals high similarity to acetyl-CoA-acetyltransferase (thiolase) accession number NP_349376 with 68% identity over 391 amino acids.

Cloning of the acetyl-CoA acetyltransferase

For cloning and expression in *E.coli* the sequence encoding the acetyl-CoA acetyltransferase was PCR amplified directly from *C. difficile* genomic DNA, and prepared using DNeasy spin columns (Qiagen) according to the manufacturer's instructions.

PCR primers used were identified and designed by direct comparison with the *C.difficile* genomic DNA sequence encoding the candidate match for the acetyl-CoA acetyltransferase, and are SEQ ID NOs: 44 and 45, synthesized by SIGMA Genosys.

Amplification was carried out using *Taq* DNA polymerase (Invitrogen) allowing direct ligation-independent cloning into the expression vector pBAD-TA (Invitrogen), adding a C-terminally fused His_6-tag to the expressed acetyl-CoA acetyltransferase under the control of the Arabinose-inducible promoter *ara*BAD. The cloning mix was transformed in to the expression strain TOP10 (Invitrogen) and recombinants were identified using SDS-PAGE and immunoblotting using a monoclonal anti-His-tag peroxidase-conjugate antibody (SIGMA). The resulting plasmid is referred to herein as pThiol1.

Expression and purification of the acetyl-CoA acetyltransferase

For over-expression of the 6-His-tag thiolase fusion protein, *E.coli* strain TOP10 (pThiol1) was grown to late exponential phase (OD_{600} 1.0 at 37 °C with shaking at 200 rpm) and protein expression was induced by the addition of Arabinose to a final concentration of 0.2%. After a further 240 minutes growth, bacteria were harvested by centrifugation (500 g, 10 minutes 4 °C) and the pellet was resuspended in lysis buffer (6 M Guanidine Hydrochloride, 50 mM Tris-HCl pH 8) at 1/20 the starting volume and solubilized by mixing for 1 hour at room temperature. Insoluble material was removed by a further centrifugation step (10000 g, 10 minutes at room temperature) and the supernatant applied to a Qiagen spin-prep Ni-NTA column following the manufacturer's instructions. The column was washed three times with 600 µl of lysis buffer followed by three washes with 600 µl of wash buffer 1 (8 M Urea, 50 mM Tris/HCl pH 8) and three times with 600 µl of wash buffer 2 (8 M Urea, 50 mM Tris/HCl pH 8, 25 mM Imidazole). The His-Tag protein was eluted from the column with 100 µl of elution buffer (8 M Urea, 50 mM Tris/HCl pH 8, 250 mM Imidazole), the elution step was repeated and both fractions pooled. Samples were analysed by SDS-PAGE.

Testing Antibody H1L1 against cloned acetyl-CoA acetyl transferase

Cloned and purified acetyl-CoA acetyl transferase was mixed 1:1 with loading buffer and DTT and put on heating block at 90 °C for 5 minutes. 5-15 µl was separated for 35 minutes on a 10% BisTris gel (Invitrogen) using NuPAGE-MES SDS running buffer (Invitrogen).

The proteins were transblotted onto Invitrolon PVDF membrane (Invitrogen Ltd, Carlsbad, CA, USA) and blocked in 5% skimmed milk in 0.1% Tween20 in 10 mM PBS for 1 hour. The membrane was incubated with H1L1 at 1:10 (concentration approximately 400 µg/ml) in 0.1% Tween20 in 10 mM PBS for 1 hour. Washed three times for 5 minutes with 0.1% Tween20 in 10 mM PBS and then incubated for one hour with S-protein HRP conjugate (Novagen) at 1:1000. The blot was developed using Sigma-Fast DAB stain and shows a strong response to the thiolase band located just under the 50 kD marker.

Therefore, antibody from patients with *C.difficile* infection is focussed against *C.difficile* acetyl-CoA acetyl transferase and synthetic antibody constructed from the dominant light and heavy chain CDRs of those patients is specific against the *C.difficile* acetyl-CoA acetyl transferase. Therefore, *C.difficile* acetyl-CoA acetyl transferase is a strong antigen and antibody H1L1 is useful in binding it.

Conserved epitope

There are three consecutive enzymes in the butanol/butyrate-producing pathway of *Clostridium acetobutylicum* which are also present on the *Clostridium difficile* chromosome. These have been found to react with a rabbit anti-serum raised against supernatant proteins from *Clostridium difficile* (Mullany P *et al.*, FEMS Microbiol Lett., 1994 Nov 15;124(1): 61-7; PMID: 8001771).

Assuming the enzymes share a conserved epitope, the only homologous region can be found at residue 9-12 in thiolase (accession number P45362) which is present in crotonase (accession nuber P45361) residues 5-8, and this conserved sequence is SEQ ID NO: 47.

***Enterococcus faecium* and *Clostridium difficile* combination drug MIC**

Pre-Assay Preparation

The organisms were cultured onto a Columbia Blood Agar plate (CBA) to achieve single colonies and incubated at 37 °C for 24 hours (*E.faecium*) or anaerobically for 48 hours (*C.difficile*).

Antimicrobial agents were prepared according to NCCLS methodology (M7-6A), giving a total of 14 concentrations for vancomycin and gentamicin and 11 concentrations for H1L1. The initial dilutions of vancomycin and gentamicin were made in dH_2O, H1L1 was initially diluted in Formulation Buffer (In 100 ml: 3.484 g Arginine (200 mM), 3.006 g Urea (0.5 M), pH 9.5). Subsequent dilutions were made in the appropriate growth medium for the organism being used. The agents were stored in aliquots at -20 °C and thawed on the day of use.

Concentrations were twice the final concentration required.

1. Medium Preparation

   E.faecium Medium - Cation-adjusted Mueller Hinton Broth (NCCLS M7-6A): To 1 litre Mueller Hinton Broth (OXOID, MHB), 2 ml of 10 mg/ml $CaCl_2$ and 500 µl of 10 mg/ml $MgCl_2$ were added

   C.difficile Medium -

(1) Supplemented Brucella Broth (NCCLS M11-A5): To 900 ml Brucella Broth powder (SIGMA), 1 ml Hemin (5 mg/ml) and 1 ml Vitamin K (1 mg/ml) were added. Post autoclaving 100 ml Lysed horse blood (5%) was added.

(2) Reinforced clostridial medium (RCM) was prepared according to the manufacturers instructions: 38 g in 1 litre dH_2O.

2. MIC Plate Preparation – Table 1

Drug 1 - vancomycin or gentamicin (final concentration range 0.0625 µg/ml to 512 µg/ml)

In a U-shaped 96 well microtitre plate: twice the required concentration of vancomycin or gentamicin (50 µl) is added left to right along the first row of the microtitre plate leaving the final well blank. This was repeated for the other rows using 2-fold decreasing concentrations of vancomycin.

Drug 2 - H1L1 antibody (final concentration range 0.25 µg/ml to 256 µg/ml)

H1L1 (50 ml) is added down the columns. Twice the required concentration is added down the first column. This was repeated for the other columns moving along the plate (left to right) 2-fold decreasing concentrations of H1L1. The last column is left blank

The final column contains 100 µl of growth medium only

Continuing concentrations of vancomycin and gentamicin were performed in the same manner as above but on a second microtitre plate. H1L1 concentrations were exactly the same as above, as were the Media only control in column 12.

Inoculum Preparation - Direct Colony Suspension

- The inoculum was prepared immediately prior to use of the bacteria.
- A direct colony suspension was made by resuspending colonies from an 18 to 24 hour (*E.faecium*) or 48 hour (*C.difficile*) agar plate into the appropriate growth medium or sterile saline.
- This was adjusted to 0.5 MacFarlands standard then diluted 1:10 in growth medium (approximately 1 x 10^7 cfu/ml) according to NCCLS M7-6A (*E.faecium*) and NCCLS M11-A5 (*C.difficile*).

Plate Inoculation

- 5 µl of the 1:10 inoculum suspension prepared as above was used to inoculate each well (final inoculum 5 x 10^4 cfu/ml).
- The plate was inoculated from well 12 to well 1.

Incubation

- The plates were incubated at 37 °C for 24 hours (*E.faecium*) or anaerobically for 48 hours (*C.difficile*).
- To check the inoculum 10 µl from the growth control was diluted in 10 ml sterile saline (1:1000), and 100 µl was plated onto a CBA plate and incubated at 37 °C for 24 hours (*E.faecium*) or anaerobically for 48 hours (*C.difficile*). Fifty colonies were equivalent to 5 x 10^4 cfu/ml.

Reading Results

The MIC was taken as the lowest concentration of drug that substantially inhibited the growth of the organism.

The FIC (fractional inhibitory concentration) was calculated for each drug by dividing the MIC in the presence of the second drug by the MIC in its absence. For each combination this produced two fractions, which were summated to produce the FICI (fractional inhibitory concentration index): synergy was defined by a value of <=0.5, indifference was defined by a value of >0.5 to <4 and antagonism was defined by a value of >= 4.0

Results

These results demonstrate synergy between H1L1 and vancomycin and gentamicin

Conclusions

These results demonstrated synergy between both (i) H1L1 and vancomycin, and (ii) H1L1 and gentamicin versus *C. difficile* 14000287 and *C.difficile* NCTC 11204. It showed synergy between Vancomycin and H1L1 in Vancomycin resistant *Enterococcus faecium*.

Growth of *Clostridium difficile* in the presence of variable short chain fatty acids (SCFA) and H1L1 antibody concentrations

SCFA Preparation

The three SCFAs used were acetate, propionate and butyrate.

Each was prepared to a concentration of 1 M as follows:

Sodium acetate FW 82.03 - 82.03 g in 1 litre dH_2O

Sodium butyrate FW 110.09 - 110.09 g in 1 litre dH_2O

Sodium propionate FW 96.06 - 96.06 g in 1 litre dH_2O

The three solutions were combined in a ratio of 70:20:10 respectively. Therefore, in the final solution the concentrations were sodium acetate 0.7 M, sodium butyrate 0.2 M and sodium propionate 0.1 M. The final concentration of total SCFA was 1 M.

From this stock solution concentrations of 5, 10, 20, 30, 40 and 50 mM total SCFA were prepared using reinforced clostridial medium (RCM, DIFCO).

Medium preparation

Reinforced clostridial medium (RCM) was prepared according to the manufacturers instructions:

38 g in 1 litre dH_2O

Growth Curve

Day before assay

Cultures were only removed from anaerobic conditions immediately prior to use. Exposure to aerobic conditions did not exceed 30 minutes

An overnight suspension of *C.difficile* was made by inoculation 10 ml of RCM medium with colonies from a 48 hour old Columbia Blood Agar plate of the organism. The suspension was incubated for 24 hours at 37 °C under anaerobic conditions.

Day of assay

Control plate

In a flat-bottomed 96 well microtitre plate control solutions (200 µl) were placed in the wells as outlined below in Table 4. Each control used 3 wells.

Test plate

In a flat-bottomed 96 well microtitre plate (rows labelled A-H, columns labelled 1-12) test solutions (100 µl) were placed in the wells as detailed below. The two test solutions, SCFA plus H1L1, give a combined volume of 200 µl. Each test used 2 wells. Wells in row

A contained 50 mM SCFA; in row B, 40 mM SCFA; in row C, 30 mM SCFA; in row D, 20 mM SCFA; in row E, 10 mM SCFA; in row F, 0.5 mM SCFA. Wells in columns 1 and 2 contained 16 µg/ml H1L1; in columns 3 and 4, 8 µg/ml H1L1; in columns 5 and 6, 4 µg/ml H1L1; in columns 7 and 8, 2 µg/ml H1L1; in columns 9 and 10, 1 µg/ml H1L1; in columns 11 and 12, 0.5 µg/ml H1L1. Row G was empty. 200 µl RCM was placed into all the wells in row H as a growth control for the plate.

Inoculum preparation

Cultures were only removed from anaerobic conditions immediately prior to use. Exposure to aerobic conditions did not exceed 30 minutes.

The overnight suspension, culture at late log phase, was used to inoculate the microtitre plates. 20 µl (approx 10% inoculum) was added to all the wells, except for the media/negative control on the control plate.

Plates were incubated at 37 °C under anaerobic conditions for 24 hours.

Optical density readings (OD_{600} nm) were made at time points zero and 24 hours. Average OD_{600} nm readings were determined.

Results

Controls - see Table 5.

Conclusions -

1. No effect of 10, 20, 30, and 40 mM of SCFA on its own, but some decrease with 50 mM of SCFA.
2. No effect with 0.5, 1, 2 4, and 8 µg/ml of H1L1 but inhibition with 16 µg/ml of H1L1.

Tests - see Table 6.

Conclusions -

Indifference with 1, 2 and 4 μg/ml of H1L1 and 5 mM of SCFA (data not shown). Synergy with 8 μg/ml of H1L1 and SCFA from 10-50 mM with optical density at 24 hours falling to a range of 0.296 - 0.330. The effect was most obvious with 16 μg/ml of H1L1 and 50 mM of SCFA.

Table 1

Drug concentrations at this stage will be half the concentration that was put into the wells (50 µl + 50 µl = 1:2 dilution).

A, B, etc. = Vancomycin or gentamicin

1, 2, 3 etc. = + H1L1

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	Media only
B	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	Media only
C	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	Media only
D	D1	D3	D4	D4	D5	D6	D7	D8	D9	D10	D11	Media only
E	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	Media only
F	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	Media only
G	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	Media only
H	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	Media only

Table 2

Species	Agent	MIC (µg/ml) of each agent		FIC (µg/ml)	FICI	Outcome
		Alone	Combination			
Clostridium difficile NCR 1104 (Brucella broth)	Vancomycin	1	0.125	0.125	0.125	Synergy
	H1L1	512	0.25	0.0004		
Enterococcus faecium	Vancomycin	128	16	0.12	0.37	Synergy
	H1L1	512	128	0.25		

Table 3

Species	Agent	MIC (µg/ml) of each agent		FIC (µg/ml)	FICI	Outcome
		Alone	Combination			
C.difficile 14000287 (Brucella broth)	Gent	8	2	0.25	0.266	Synergy
	H1L1	512	8	0.0156		
C.difficile NCTC 11204 (RCM)	Gent	16	4	0.25	0.375	Synergy
	H1L1	512	64	0.125		

Table 4

	1	2	3	4	5	6	7	8	9	10	11	12
A	RCM no inoculum			RCM + inoculum								
B	RCM + 50 mM SCFA			RCM + 40 mM SCFA			RCM + 30 mM SCFA			RCM + 20 mM SCFA		
C	RCM + 10 mM SCFA			RCM + 5 mM SCFA								
D	RCM + 16 µg/ml H1L1			RCM + 8 µg/ml H1L1			RCM + 4 µg/ml H1L1			RCM + 2 µg/ml H1L1		
E	RCM + 1 µg/ml H1L1			RCM + 0.5 µg/ml H1L1								
F												
G												
H												

Table 5

Experiment No	H1L1 (μg/ml)	SCFA (mM)	Mean Optical Density at 24 Hours
1	-	-	0.452
2	-	5	0.460
3	-	10	0.429
4	-	20	0.452
5	-	30	0.480
6	-	40	0.440
7	-	50	0.370
8	0.5	-	0.450
9	1	-	0.440
10	2	-	0.440
11	4	-	0.440
12	8	-	0.420
13	16	-	0.334

Table 6

Experiment No	H1L1 (μg/ml)	SCFA (mM)	Mean Optical Density at 24 Hours
1	8	10	0.310
2	16	10	0.308
3	8	20	0.296
4	16	20	0.228
5	8	30	0.305
6	16	30	0.308
7	8	40	0.310
8	16	40	0.303
9	8	50	0.330
10	16	50	0.281

CLAIMS

1. The use of an inhibitor of acetyl-CoA acetyltransferase in the manufacture of a medicament for the treatment or prophylaxis of infection by *Clostridium difficile*.

2. The use according to claim 1, said acetyl-CoA acetyltransferase being from *Clostridium difficile*.

3. The use according to either of the preceding claims, said inhibitor being an antibody or an antigen binding fragment thereof.

4. The use according to claim 3, said antibody or antigen binding fragment thereof being specific against an epitope displayed by the peptide having the sequence of SEQ ID NO: 47.

5. The use according to either of claims 3 or 4, said antibody or antigen binding fragment thereof having VH chain complementarity determining regions (CDRs) 1-3 having the sequences of SEQ ID NOs: 2-4 respectively.

6. The use according to claim 5, said antibody or antigen binding fragment thereof VH chain having the sequence of SEQ ID NO: 1.

7. The use according to any of claims 3-6, said antibody or antigen binding fragment thereof having VL chain complementarity determining regions (CDRs) 1-3 having the sequences of SEQ ID NOs: 17-19.

8. The use according to claim 7, said antibody or antigen binding fragment thereof VL chain having the sequence of SEQ ID NO: 16.

9. The use according to claim 3, said antibody or antigen binding fragment thereof having the sequence of SEQ ID NO: 41.

10. The use according to any of the preceding claims, the medicament additionally comprising at least one of the group of antibiotics consisting: gentamicin, vancomycin and metronidazole.

11. A combined preparation for the treatment of infection by *Clostridium difficile*, comprising:

(i) an inhibitor of acetyl-CoA acetyltransferase; and

(ii) at least one of the group of antibiotics consisting: gentamicin, vancomycin and metronidazole.

12. Isolated and/or purified antibody or antigen binding fragment thereof comprising at least one of the group consisting of:

(i) VH chain complementarity determining regions (CDRs) 1-3 having the sequences of SEQ ID NOs: 2-4 respectively; and

(ii) VL chain complementarity determining regions (CDR) 3 having the sequence of SEQ. ID NO: 19.

13. Isolated and/or purified antibody or antigen binding fragment thereof according to claim 12, said VH chain having the sequence of SEQ ID NO: 1.

14. Isolated and/or purified antibody or antigen binding fragment thereof according to either of claims 12 or 13, said VL chain having the sequence of SEQ ID NO: 16.

15. Isolated and/or purified antibody or antigen binding fragment thereof according to claim 12, said antibody having the sequence of SEQ ID NO: 41.

16. A nucleic acid molecule coding for an antibody or antigen binding fragment thereof according to claim 15.

17. A nucleic acid molecule according to claim 16, having the sequence of SEQ ID NO: 46.

18. The use of an inhibitor of acetyl-CoA acetyltransferase and vancomycin in the manufacture of a medicament for the prophylaxis or treatment of infection by *Enterococcus faecium* and *Enterococcus faecalis*.

19. The use according to claim 8, said *Enterococcus faecium* or *Enterococcus faecalis* being vancomycin resistant.

20. The use according to either of claims 18 or 19, said acetyl-CoA acetyltransferase being from *Clostridium difficile*.

21. The use according to any of claims 18-20, said inhibitor being an antibody or an antigen binding fragment thereof.

22. The use according to claim 21, said antibody or antigen binding fragment thereof being specific against an epitope displayed by the peptide having the sequence of SEQ ID NO: 47.

23. The use according to either of claims 21 or 22, said antibody or antigen binding fragment thereof having VH chain complementarity determining regions (CDRs) 1-3 having the sequences of SEQ ID NOs: 2-4 respectively.

24. The use according to claim 23, said antibody or antigen binding fragment thereof VH chain having the sequence of SEQ ID NO: 1.

25. The use according to any of claims 21-24, said antibody or antigen binding fragment thereof having VL chain complementarity determining regions (CDR) 3 having the sequence of SEQ. ID NO: 19.

26. The use according to claim 25, said antibody or antigen binding fragment thereof VL chain having the sequence of SEQ ID NO: 16.

27. The use according to claim 21, said antibody or antigen binding fragment thereof having the sequence of SEQ ID NO: 41.

28. A combined preparation for the treatment of infection by *Enterococcus faecium* or *Enterococcus faecalis*, comprising:

(i) an inhibitor of acetyl-CoA acetyltransferase; and

(ii) vancomycin.

29. A method of treatment of infection by *Clostridium difficile*, comprising administering a therapeutically effective quantity of an inhibitor of acetyl-CoA acetyltransferase to a patient in need of same.

30. A method according to claim 29, additionally comprising administering a therapeutically effective quantity of at least one of the group of antibiotics consisting: gentamicin, vancomycin and metronidazole.

31. A method of treatment of infection by *Enterococcus faecium* or *Enterococcus faecalis*, comprising administering a therapeutically effective quantity of:

(i) an inhibitor of acetyl-CoA acetyltransferase; and

(ii) vancomycin

to a patient in need of same.

32. A method according to claim 31, said *Enterococcus faecium* or *Enterococcus faecalis* being vancomycin resistant.

SEQUENCE LISTING

<110> NeuTec Pharma Plc
Burnie, James
Matthews, Ruth
Carter, Tracey

<120> Treatment of Bacterial Infections

<130> NA/MP101123WO

<150> GB 0414886.2
<151> 2004-07-02

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Gly Lys Asn Asn Arg Pro Ser Gly Ile Pro Asp Arg Phe Ser Gly Ser
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35 40 45

Trp Ile Gly Phe Ile Tyr Tyr Thr Gly Tyr Thr Ser Tyr Lys Ser Ser
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Leu Lys Ser Arg Val Ser Leu Ser Val Asp Thr Ser Asn Asp Gln Phe
65 70 75 80

Ser Leu Ser Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val Tyr Tyr
85 90 95

Cys Ala Arg Glu Ile Arg Ala Pro Asp His His Asp Phe Ser Gly Tyr

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TRATAMIENTO DE INFECCIONES BACTERIANAS

CAMPO DE LA INVENCION

La presente invención se refiere a compuestos, medicamentos y tratamientos para infección por *Clostridium difficile*, junto con anticuerpos aislados novedosos y su uso en la misma. La presente invención también se refiere a la profilaxis y tratamiento de infección por *E. faecium* y *E. faecalis* y provee medicamentos y tratamientos para las mismas.

ANTECEDENTES DE LA INVENCION

Clostridium difficile es una bacteria anaeróbica Gram positiva, y es considerada como un patógeno humano que ocasiona un espectro de enfermedades que varían desde diarrea leve hasta colitis pseudomembranosa fulminante (PMC por sus siglas en inglés)- denominadas colectivamente como diarrea asociada con antibiótico contra *Clostridium difficile* (CDAD por sus siglas en inglés). CDAD es una enfermedad nosocómica, iatrogénica, común asociada con mortalidad y morbilidad sustanciales, especialmente en los adultos mayores. A dos de los factores se les han asignado papeles principales en la patogénesis de CDAD - la

supresión de la flora intestinal residente por la administración de antibióticos, y la producción por parte de la bacteria de dos toxinas de alto peso molecular, exotoxina A y exotoxina B.

La bacteria es endémica en hospitales, y los estudios han demostrado que aproximadamente un tercio de los pacientes que reciben tratamiento con antibiótico en pabellones médicos de cuidado intensivo son colonizados con *Clostridium difficile* mientras están en el hospital (Kyne, L., *et al.*, 2002, Clin. Infect. Dis. 34(3), pp346-53, PMID: 11774082). De estos pacientes, casi la mitad desarrollan CDAD mientras el resto son portadores asintomáticos. CDAD es un factor importante en la extensión del tiempo de permanencia de los pacientes en el hospital, y las estimaciones sugieren que el costo de esta enfermedad en los Estados Unidos de Norteamérica supera los 1,100 millones de dólares por año (Kyne, L., *et al.*, *supra*). Los pacientes que padecen de CDAD responden bien a un tratamiento que incluya suspender el antibiótico incitador y el tratamiento con cualquiera de los antibióticos metronidazol y vancomicina. La tasa de respuesta a la terapia inicial con antibiótico puede ser alta (hasta 95%), pero hasta un 20% de los pacientes pueden presentar relapso dentro de un tiempo de una a dos semanas de un primer tratamiento, con el riesgo de que el relapso se agrave con

cada episodio de relapso. El relapso se puede tratar con antibióticos, lo que indica que éste se debe a una infección con cepas diferentes de *C. difficile*. De manera adicional, existe evidencia que *C. difficile* se está haciendo resistente a metronidazol y parcialmente resistente a vancomicina, lo que demuestra la necesidad respecto a nuevas alternativas en el tratamiento de CDAD.

SUMARIO DE LA INVENCION

Las exotoxinas A y B las cuales son producidas por cepas patogénicas de la bacteria son citotóxicas, enterotóxicas y pro-inflamatorias, y se considera que son las factores de virulencia principales de este microorganismo no invasor. Sin embargo, no todas las infecciones con cepas toxigénicas dan como resultado la enfermedad, lo cual impulsa la búsqueda respecto a factores de virulencia adicionales. Los antígenos expresados en la superficie bacteriana representan candidatos para factores de virulencia, y también se consideran importantes debido a que dichas proteínas probablemente median funciones esenciales tales como adhesión a las capa epitelial del intestino en el primer paso de colonización o interacción con mediadores de inmunidad local. En común con muchas otras bacterias, *C. difficile* expresa una capa de

superficie cristalina o para-cristalina (capa S) sobre la superficie celular exterior. Dichas capas S comprenden proteínas o glucoproteínas que forman una red ordenada en forma regular sobre la superficie externa de la bacteria, y se ha demostrado previamente que son esenciales para la virulencia de patógenos tales como *Aeromanas salmonicida* y *Campylobacter fetus*. En contraste con la mayoría de bacterias que comprenden una capa S, se sabe que *C. difficile* comprende dos capas S paracristalinas sobrepuestas, cada una constituida por una subunidad de glucoproteína que varía ligeramente en el peso molecular aparente entre cepas diferentes de *C difficile*. La mayoría de cepas de *C. difficile* expresan dos proteínas principales de capa S (SLP por sus siglas en inglés), una de 32-38 kDa (SLP de bajo peso molecular) y una segunda proteína de 42-48 kDa (SLP de alto PM). La SLP de bajo PM parece ser inmuno-dominante y es el antígeno más comúnmente reconocido por pacientes que padecen de CDAD, y es el único antígeno reconocido en extractos con EDTA de bacterias utilizando antisueros generados en conejos contra células intactas de *C. difficile* (Calabi, E. *et al.*, 2001 , Mol. Microbiol., 40(5) p1187-99, PMID: 11401722).

Durante el curso de infección microbiana el sistema inmune utiliza varias estrategias de adaptación. Una de dichas estrategias, y discutiblemente la más

importante, es la producción de una respuesta de anticuerpo. Se producen y se unen anticuerpos que puedan unirse a los antígenos desplegados por el agente infeccioso y éstos permiten la aniquilación de los micro-organismos a través de activación del complemento, reclutamiento de macrófago y a través de la interacción directa con el microbio mismo. La eficacia terapéutica de los anticuerpos que tienen capacidad de unir un antígeno dado varía y esto se refleja en el hecho que la producción de anticuerpo por parte del sistema inmune madura durante el curso de una infección y se torna enfocada en el caso de un paciente que lucha exitosamente contra una infección.

La respuesta de anticuerpo es inducida por el repertorio de célula B en el cual células B individuales producen cada una moléculas de anticuerpo estructuralmente diversas. Se desconoce el tamaño real de este repertorio de célula B/anticuerpo, pero se calcula que la frecuencia de reactividad de clones aleatoria para un antígeno dado puede ser tan alta como de 1 en 100,000 en células B en cultivo (Nobrega, A., et al., Eur J Immunol., abril de 1998;28(4):1204-15; PMID: 9565360). Durante el curso de la infección, los anticuerpos que pueden unirse al patógeno se seleccionan mediante cambios en la población de células B lo que da como resultado que se produzcan números grandes de anticuerpos clave. Los mecanismos para estos cambios

incluyen expansión clonal, conmutación de isotipo, y mutación somática de regiones variables de inmunoglobulina. Se multiplican las células B responsables de generar anticuerpos que puedan unirse a un patógeno, alterando por lo tanto el repertorio de células B y cambiando las proporciones de células B.

Se descubre que los anticuerpos específicos contra dominio de unión a célula de la exotoxina A de *C. difficile* son protectores en un modelo de ratón, y se encuentra que los pacientes colonizados con *C. difficile* pero asintomáticos para éste tienen un título elevado de IgG anti-exotoxina A. Los pacientes infectados que desarrollan dichos títulos elevados de IgG anti-exotoxina A en suero en respuesta a la colonización tienen una probabilidad 48 veces menor de padecer de CDAD que los pacientes que no desarrollan dicho título elevado. Por lo tanto, se sugiere la vacunación con exotoxina A de *C. difficile* como una estrategia terapéutica, al igual que el uso de anticuerpos anti-exotoxina A de *C. difficile* (Giannasca PJ y Wamy M., Vaccine, 17 de febrero de 2004;22(7): 848-56; PMID: 15040937).

Los regímenes terapéuticos no basados en antibiótico para el tratamiento/prevención de infección por *C. difficile* se basan en la vacunación e inmunización pasiva. El tratamiento de vacunación comprende administrar

a un paciente ya sea una secuencia de ácido nucleico que codifica para un fragmento inmunogénico de la proteína de capa de superficie de *C. difficile* o una variante u homólogo de la misma, o un fragmento polipeptídico equivalente (como se describe en el documento WO 02/062379). La inmunoterapia pasiva típicamente se logra administrando a un paciente un anticuerpo monoclonal específico para un inmunógeno producido por un patógeno. En general, la inmunoterapia pasiva es particularmente efectiva para tratar pacientes inmunocomprometidos que son incapaces de responder a la vacunación, y para pacientes que necesitan terapia inmediata y que no pueden esperar a que la vacunación surta efecto. En el caso de una infección por *C. difficile*, la inmunización pasiva se basa en administrar a un paciente inmunoglobulina policlonal neutralizante de la toxina (como se describe en el documento WO 99/20304), o anticuerpos generados contra la bacteria intacta y las toxinas (como se describe en el documento WO 96/07430).

Por lo tanto, el tratamiento efectivo de la infección por *C. difficile* es bastante importante, y la técnica antecedente enseña que el objetivo para dicha terapia debe ser la vacunación o inmunoterapia pasiva dirigida contra la exotoxina A de *C. difficile*, contra la proteína de capa de superficie, o como un suero policlonal

específico contra toxinas bacterianas no identificadas.

Sin embargo, el inventor de la presente invención ha identificado un objetivo específico para terapia. Los experimentos descritos más adelante muestran que los pacientes infectados con *C. difficile* producen un título elevado de anticuerpos específicos contra acetil-CoA acetiltransferasa (tiolasa), y que dicho anticuerpo anti-acetil-CoA acetiltransferasa (tiolasa) *per se* es útil y efectivo para tratar la infección por *C. difficile*. Asimismo, el anticuerpo anti-acetil-CoA acetiltransferasa, particularmente un anticuerpo sintético que se construye a partir de las secuencias de CDR más dominantes provenientes de pacientes infectados con *C. difficile*, cuando se utilizan junto con los antibióticos vancomicina o gentamicina, da como resultado la terapia sinergista de la infección por *C. difficile*.

DESCRIPCION DETALLADA DE LA INVENCION

Por lo tanto, de conformidad con un primer aspecto de la presente invención, se provee el uso de un inhibidor de acetil-CoA acetiltransferasa en la fabricación de un medicamento para el tratamiento o profilaxis de infección por *Clostridium difficile*.

Tal como se utiliza en la presente invención, el

término "tratamiento" pretende tener un significado amplio a menos que se indique explícitamente lo contrario. Por lo tanto, con "tratamiento" o "terapia" se quiere decir cualquier tratamiento que esté diseñado para curar, aliviar, eliminar o aminorar los síntomas, o para prevenir o reducir la posibilidad de contraer trastornos o disfunciones del cuerpo humano o animal. Por lo tanto, con el término "tratamiento" se quiere decir tanto tratamiento de condiciones patológicas, así como su profilaxis.

En particular, la acetil-CoA acetiltransferasa puede provenir de *Clostridium difficile*, y ésta puede tener la secuencia de SEQ ID NO: 43.

Se puede utilizar una amplia gama de inhibidores los cuales son específicos contra la acetil-CoA acetiltransferasa. En particular, la acetil-CoA acetiltransferasa puede formar un par de unión específico (sbp por sus siglas en inglés) con el inhibidor, en el que la acetil-CoA acetiltransferasa es el primer miembro del par, y el inhibidor es el segundo miembro.

En la presente invención, un "miembro de un par de unión específico" es una de dos moléculas diferentes, que tiene un área sobre la superficie o en una cavidad que se una específicamente a y por lo tanto se define como complementaria con una organización polar y espacial particular de la otra molécula. Los miembros del par de

unión específico son conocidos como ligando y receptor (anti-ligando), miembro de sbp y compañero de sbp, miembros de sbp o similares. Estos son normalmente miembros de un par inmunológico tal como antígeno-anticuerpo, aunque el término tiene un significado más amplio que abarca otros pares de unión específicos, tales como ARN-proteína, ADN-proteína, y similares.

Por lo tanto, el inhibidor puede ser un anticuerpo o fragmento de unión a antígeno del mismo, y éste puede ser específico contra un epítope desplegado por el péptido de SEQ ID NO: 47.

Como se describe con mayor detalle más adelante en la Sección de Experimentos, se ha identificado que el epítope desplegado por el péptido que tiene la secuencia de SEQ ID NO: 47 se conserva entre varios antígenos y por lo tanto se ha identificado como el péptido que despliega el epítope conservado compartido por dichos antígenos.

El término "anticuerpo" en sus diversas formas gramaticales se utiliza en la presente invención para hacer referencia a moléculas de inmunoglobulina y a porciones inmunológicamente activas de moléculas de inmunoglobulina, es decir, moléculas que contienen un sitio de combinación a anticuerpo o paratope. Dichas moléculas también son conocidas como "fragmentos de unión a antígeno" de las moléculas de inmunoglobulina.

Las moléculas de anticuerpo ilustrativas son moléculas de inmunoglobulina intactas, moléculas de inmunoglobulina sustancialmente intactas y aquellas porciones de una molécula de inmunoglobulina que contienen el paratope, incluyendo aquellas porciones conocidas en la técnica como Fab, Fab', $F(ab')_2$, scFv y F(v). Los anticuerpos, su producción y su uso son bien conocidos en la técnica (por ejemplo Harlow, E. y Lane, D., "Using Antibodies: A Laboratory Manual", Cold Spring Harbor Laboratory Press, New York, 1998).

Como se describe con mayor detalle más adelante en los experimentos, se clonan y se determina la secuencia de las cadenas pesada variable (VH) y ligera variable (VL) de los anticuerpos producidos por pacientes infectados con *C. difficile* y se identifican sus regiones determinantes de complementariedad (CDR por sus siglas en inglés). Esto demuestra que existen 1-3 CDRs de cadena VH altamente inmuno-dominantes (CDR-H1, CDR-H2 y CDR-H3), y 1-3 CDRs de cadena VL altamente inmuno-dominantes (CDR-L1, CDR-L2 y CDR-L3).

Por lo tanto, el anticuerpo o fragmento de unión a antígeno del mismo puede tener 1-3 regiones determinantes de complementariedad (CDRs) de cadena VH que tengan las secuencias de SEQ ID NOs: 2-4 respectivamente. En particular, el anticuerpo o fragmento de unión a antígeno

del mismo puede tener una cadena VH que tenga la secuencia de SEQ ID NO: 1.

De manera similar, el anticuerpo o fragmento de unión a antígeno del mismo puede tener 1-3 regiones determinantes de complementariedad (CDRs) de cadena VL que tengan las secuencias de SEQ ID NOs: 17- 19. En particular, el anticuerpo o fragmento de unión a antígeno del mismo puede tener una cadena VL que tenga la secuencia de SEQ ID NO: 16.

Se crea y se utiliza un anticuerpo sintético que tiene las secuencias anteriores, y por lo tanto el anticuerpo puede tener la secuencia de SEQ ID NO: 41. El anticuerpo descrito más adelante con mayor detalle que tiene SEQ ID NO: 41 también comprende una marca S N-terminal y una marca His C-terminal. Las marcas son útiles para propósitos experimentales pero son innecesarias para el anticuerpo terapéutico, aunque éstas (u otras marcas) se pueden considerar útiles. Por lo tanto, por ejemplo, el anticuerpo puede comprender de manera adicional una marca His, por ejemplo una marca His C-terminal.

Los experimentos más adelante han demostrado que no solamente es un inhibidor de acetil-CoA acetiltransferasa efectivo por sí mismo para efectuar el tratamiento de infección por *Clostridium difficile*, sino que también es útil cuando se utiliza en conjunto con

antibióticos existentes, en particular gentamicina y vancomicina, y que dicho uso puede proveer resultados sinergistas. Los resultados experimentales preliminares también demuestran que se obtiene sinergismo con metronidazol.

Por lo tanto, el medicamento puede comprender de manera adicional por lo menos uno del grupo de antibióticos que consiste de: gentamicina, vancomicina y metronidazol.

Desde luego, las formulaciones médicas de la presente invención pueden comprender un vehículo, diluyente o excipiente farmacéuticamente aceptable, (Remington's Pharmaceutical Sciences y Farmacopea de EE.UU, 1984, Mack Publishing Company, Easton, PA, EE.UU; Farmacopea de los Estados Unidos de Norteamérica, ISBN: 1889788031).

Como se describió en forma detallada anteriormente, un inhibidor de acetil-CoA acetiltransferasa efectivo por si mismo para efectuar el tratamiento de infección por *Clostridium difficile*. Por lo tanto, de conformidad con la presente invención también se provee un anticuerpo aislado y/o purificado que comprende por lo menos uno del grupo que consiste de:

(i) 1-3 regiones determinantes de complementariedad (CDRs) de cadena VH que tengan las secuencias de SEQ ID NOs: 2-4 respectivamente; y

(ii) 1-3 regiones determinantes de

complementariedad (CDRs) de cadena VL que tengan las secuencias de SEQ ID NOs: 17-19.

Como se indicó anteriormente, la cadena VH puede tener la secuencia de SEQ ID NO: 1. La cadena VL puede tener la secuencia de SEQ ID NO: 16. El anticuerpo puede tener la secuencia de SEQ ID NO: 41.

También se provee una molécula de ácido nucleico que codifica para dicho anticuerpo. La molécula de ácido nucleico puede ser aislada y/o purificada. En particular, la molécula de ácido nucleico puede tener la secuencia de SEQ ID NO: 46.

También se provee de conformidad con la presente invención el uso de un inhibidor de acetil-CoA acetiltransferasa y vancomicina en la fabricación de un medicamento para la profilaxis o tratamiento de infección por *Enterococcus faecium*, o *Enterococcus faecalis*, en particular *Enterococcus faecium* resistente a vancomicina.

Los siguientes experimentos muestran que el uso de un inhibidor de acetil-CoA acetiltransferasa y vancomicina no solo da como resultado la terapia sinergista de infección por *C. difficile*, sino que también da como resultado la terapia sinergista de infección por *E. faecium*, en particular infección por *E. faecium* resistente a vancomicina. Los experimentos preliminares también han demostrado que el sinergismo también se observa en el

tratamiento de infección por *E. faecalis*.

La acetil-CoA acetiltransferasa puede ser de *Clostridium difficile*.

El inhibidor puede ser un anticuerpo o fragmento de unión a antígeno del mismo, y éste puede ser específico contra un epítope desplegado por el péptido que tiene la secuencia de SEQ ID NO: 47.

Al igual que con otros aspectos de la presente invención, el anticuerpo o fragmento de unión a antígeno del mismo puede tener 1-3 regiones determinantes de complementariedad (CDRs) de cadena VH que tengan las secuencias de SEQ ID NOs: 2-4 respectivamente. La cadena VH del anticuerpo o fragmento de unión a antígeno del mismo puede tener la secuencia de SEQ ID NO: 1.

El anticuerpo o fragmento de unión a antígeno del mismo puede tener 1-3 regiones determinantes de complementariedad (CDRs) de cadena VL que tengan las secuencias de SEQ ID NOs: 17-19. La cadena VL del anticuerpo o fragmento de unión a antígeno del mismo puede tener la secuencia de SEQ ID NO: 16.

El anticuerpo o fragmento de unión a antígeno del mismo puede tener la secuencia de SEQ ID NO: 41.

En casos en los que la presente invención se refiere a la provisión de medicamentos que contienen dos o más ingredientes activos (por ejemplo anticuerpo y un

antibiótico), la presente invención también provee preparaciones combinadas que comprenden dichos ingredientes activos. Por ejemplo, se pueden proveer preparaciones de dos paquetes.

Por lo tanto, de conformidad con la presente invención también se provee una preparación combinada para el tratamiento de infección por *Clostridium difficile*, que comprende:

(i) un inhibidor de acetil-CoA acetiltransferasa; y

(ii) por lo menos uno del grupo de antibióticos que consiste de: gentamicina, vancomicina, y metronidazol.

También se provee una preparación combinada para el tratamiento de infección por *Enterococcus faecium* o *Enterococcus faecalis*, que comprende:

(i) un inhibidor de acetil-CoA acetiltransferasa; y

(ii) vancomicina.

La *Enterococcus faecium* o *Enterococcus faecalis* puede ser resistente a vancomicina.

La presente invención también se extiende a métodos de tratamiento de pacientes. Por lo tanto, de conformidad con la presente invención también se provee un método de tratamiento de infección por *Clostridium difficile*, que comprende administrar una cantidad terapéuticamente efectiva de un inhibidor de acetil-CoA acetiltransferasa a un paciente en necesidad del mismo. El

método puede comprender de manera adicional administrar una cantidad terapéuticamente efectiva de por lo menos uno del grupo de antibióticos que consiste de: gentamicina, vancomicina y metronidazol.

También se provee de conformidad con la presente invención un método de tratamiento de infección por *Enterococcus faecium* o *Enterococcus faecalis*, que comprende administrar una cantidad terapéuticamente efectiva de:

(i) un inhibidor de acetil-CoA acetiltransferasa; y

(ii) vancomicina

a un paciente en necesidad del mismo.

La *Enterococcus faecium* puede ser resistente a vancomicina.

La invención será evidente adicionalmente a partir de la siguiente descripción, la cual muestra, únicamente a manera de ejemplo, formas de tratamiento de infección por *C. difficile* y *E. faecium*.

EXPERIMENTOS

En los siguientes experimentos, se construye un anticuerpo sintético utilizando las secuencias de anticuerpo de VH y VL más predominantes provenientes de pacientes infectados con *C. difficile*. Utilizando este anticuerpo sintético, se aísla y purifica un antígeno y se

utiliza espectrometría de masas por electroaspersión para determinar una secuencia parcial putativa para la proteína aislada. Al investigar las secuencias genómicas de *C. difficile* respecto a coincidencias se identifica una posible secuencia candidato coincidente. Una comparación de la secuencia candidato de *C. difficile* identifica un número de proteínas homólogas que se clasifican como enzimas de tipo acetil-CoA acetiltransferasa (tiolasa) y confirma a ésta como un miembro de dicha familia. La coincidencia más cercana es NP_349376 de *Clostridium acetobutylicum*, la cual demuestra un 68% de homología a lo largo de 391 aminoácidos. La clonación, expresión y purificación de la proteína candidato producen una proteína que se une fuertemente con el anticuerpo sintético. Los experimentos adicionales han demostrado que el anticuerpo específico contra la acetil-CoA acetiltransferasa (tiolasa) de *C. difficile* muestra sinergismo con vancomicina y gentamicina al tratar infecciones por *C. difficile*. Los experimentos preliminares también muestran que también se puede obtener sinergismo con metronidazol.

Los experimentos también muestran sinergismo entre el anticuerpo sintético y vancomicina en el tratamiento de *E. faecium* resistente a vancomicina. Los experimentos preliminares (no mostrados) también indican sinergismo entre el anticuerpo sintético y vancomicina en

el tratamiento de infección por *E. faecalis*.

A menos que se indique de otra manera, todos los procedimientos se efectúan utilizando protocolos estándar y siguiendo las instrucciones del fabricante en casos en los que sea aplicable. Los protocolos estándar para diversas técnicas incluyendo PCR, clonación molecular, manipulación y determinación de secuencia, fabricación de anticuerpos, mapeo de epítope y diseño de mimótope, cultivo celular y despliegue de fago, se describen en textos tales como McPherson, MJ. *et al.* (1991, PCR: A practical approach, Oxford University Press, Oxford), Sambrook, J. *et al.* (1989, Molecular cloning: a laboratory manual, Cold Spring Harbour Laboratory, New York), Huynh y Davies (1985, "DNA Cloning Vol I - A Practical Approach", IRL Press, Oxford, Ed. D.M. Glover), Sanger, F. *et al.* (1977, PNAS USA 74(12): 5463-5467), Harlow, E. y Lane, D. ("Using Antibodies: A Laboratory Manual", Cold Spring Harbor Laboratory Press, New York, 1998), Jung, G. y Beck-Sickinger, A.G. (1992, Angew. Chem. Int. Ed. Eng., 31; 367-486), Harris, M.A. y Rae, LF. ("General Techniques of Cell Culture", 1997, Cambridge University Press, ISBN 0521 573645), "Phage Display of Peptides and Proteins: A Laboratory Manual" (Eds. Kay, B.K., Winter, J., y McCafferty, J., Academic Press Inc., 1996, ISBN 0-12-402380-0).

Los reactivos y equipo útiles en, entre otros,

los métodos descritos con detalle en la presente invención se pueden conseguir a partir de los similares de Amersham (www.amersham.co.uk), Boehringer Mannheim (www.boehringer-ingeltheim.com), Clontech (www.clontech.com), Genosys (www.genosys.com), Millipore (www.millipore.com), Novagen (www.novagen.com), Perkin Elmer (www.perkinelmer.com), Pharmacia (www.pharmacia.com), Promega (www.promega.com), Qiagen (www.qiagen.com), Sigma (www.sigma-aldrich.com) y Stratagene (www.stratagene.com).

Los contenidos de cada una de las referencias discutidas en la presente invención, incluyendo las referencias citadas en las mismas, quedan incorporadas en la presente invención para referencia en su totalidad.

En casos en los que se dan números de referencia "PMID:" para publicaciones, estos son los números de identificación de PubMed asignados a éstos por la Biblioteca Nacional de Medicina de EE.UU, a partir de los cuales se puede conseguir la información bibliográfica completa y el resumen para cada publicación en www.ncbi.nlm.nih.gov. Estos también pueden proveer acceso directo a copias electrónicas de las publicaciones, en particular en el caso de por ejemplo publicaciones de PNAS, JBC y MBC.

La homología de secuencia es como se determina utilizando el programa BLAST2 (Tatusova TA *et al.*, FEMS

Microbiol Lett., mayo de 1999 15;174(2):247-50; PMID: 10339815) en el Centro Nacional para Información sobre Biotecnología, E.U.A. (www.ncbi.nlm.nih.gov) con los parámetros predeterminados.

Identificación de las secuencias de anticuerpo de VH y VL dominantes provenientes de pacientes infectados con *C. difficile*

Con el fin de identificar las secuencias de anticuerpo provenientes de pacientes con infecciones de *C. difficile* y determinar las secuencias de anticuerpo dominante, en particular secuencias de CDR, se utilizan los métodos descritos con detalle en el documento WO 03/052416. Se identifican las células B que producen anticuerpos, se les determina la secuencia y se analizan, utilizando los siguientes pasos básicos (véase documento WO 03/052416):

(1) Aislamiento de las regiones codificadoras de VH y/o VL a partir de células B en circulación de pacientes humanos.

(2) Determinar la secuencia de nucleótido de los repertorios de VH y/o VL.

(3) Determinación de las secuencias de aminoácido primarias de VH y/o VL.

(4) Extracción de las regiones CDR *in silico* -

incorporación en la base de datos.

(5) Detección de las regiones CDR y de estructura básica en el repertorio VH y/o VL.

(6) Construcción y producción de anticuerpos recombinantes terapéuticos a partir de las secuencias de anticuerpo dominante.

Se determina la secuencia de los fragmentos de anticuerpos provenientes de cuatro pacientes infectados (D01, D02, D03 y D04).

Cadena pesada (CDH1)

CDH1 es la cadena VH que se presenta de manera más común proveniente de los pacientes infectados con *C. difficile*. Esta se identifica a partir del análisis de las secuencias de CDR3 proveniente de las cadenas VH del anticuerpo del paciente. 226/1011 (22.4%) de los anticuerpos clonados provenientes de pacientes infectados con *C. difficile* tienen la misma secuencia de CDR3 (CDR-H3, más adelante). Se encuentra que CDH1 está presente en tres cuartas partes de los pacientes. Su secuencia de CDR3 aparece en 184/318 (57.9%) de los clones provenientes del paciente D01; en D03 éste aparece en 40/291 (13.7%) de los clones; y en D04 éste aparece en 2/252 (0.8%) de los clones. La secuencia de VH completa más común es SEQ ID NO: 1.

Dentro de esta secuencia de cadena VH, las secuencias de CDR (región determinante de complementariedad) son las siguientes:

CDR-H1	SEQ ID NO:2
CDR-H2	SEQ ID NO:3
CDR-H3	SEQ ID NO:4

Las investigaciones de homología han identificado algunas otras secuencias de CDR3 que tienen más de 70% de homología con la secuencia de CDR-H3 (SEQ ID NO: 4), todas provenientes de pacientes con *C. difficile*. Las secuencias de CDR3 son SEQ ID NOs: 5-15.

Cadena ligera (CDL1)

La cadena ligera para H1L1 se obtiene a partir de un clon con la secuencia de CDR3 más común proveniente de pacientes infectados con *C. difficile*. CDL1 está presente en el paciente D01 con una frecuencia de 84/251 (33.5%). La secuencia de VL más común que contiene esta secuencia de CDR3 es SEQ ID NO: 16.

Dentro de esta secuencia de cadena de VL, las secuencias de CDR (región determinante de complementariedad) son las siguientes:

CDR-L1	SEQ ID NO:17
CDR-L2	SEQ ID NO:18
CDR-L3	SEQ ID NO:19

Las investigaciones de homología han identificado algunas otras secuencias de CDR3 que tienen más de 70% de homología con la secuencia de CDR-L3, todas provenientes de pacientes con *C. difficile*. Las secuencias de CDR3 son SEQ ID NOs: 20-40.

Construcción y clonación del anticuerpo H1L1

El anticuerpo sintético H1L1 se construye de la siguiente manera: se amplifican mediante PCR por separado CDH1 y CDL1 a partir del vector para determinación de secuencia y se clonan en el vector de clonación "pGEM-T easy" (Promega Corporation) para facilitar la determinación de secuencia de ADN. Para esto se preparan 3 µg del producto de PCR para restricción utilizando columnas de centrifugación para purificación mediante PCR QIAquick (Qiagen, UK) de conformidad con las instrucciones del fabricante. El ADN se diluye a partir de la columna de centrifugación en 40 µL de solución reguladora EB. El producto de PCR purificado (2 µL) se mezcla con 1 µl del vector pGEM-T easy, 6 µl de agua y 1 µl de ADN ligasa y la mezcla se liga durante 1 hora a temperatura ambiente. Las

ligaciones se transforman después en células TG1 de *E. coli* electro-competentes (Stratagene) mediante electroporación, y se siembran en placas de agar que contienen 100 µg/ml de ampicilina, IPTG (100 µM) y X-gal (0.006 % p/v). Se permite que las colonias crezcan durante la noche a 37°C y después se almacenan a 4°C. Las colonias recombinantes se identifican como colonias de color blanco en este medio.

Esto produce un anticuerpo que tiene la estructura general:

Marca S - CDH1 - Enlazador - CDL1 - Marca His

que tiene la secuencia de aminoácido de SEQ ID NO: 41 con una marca S N-terminal y una marca His C- terminal.

Identificación del objetivo para el anticuerpo H1L1 - acetil-CoA acetiltransferasa (Tiolasa)

Materiales y métodos

Preparación de la muestra

Se suspenden células de *Clostridium difficile* (NCTC 11204) cultivadas en placas de agar sangre en 10 mM de PBS (solución salina regulada de fosfato) y se les irradia con energía sónica durante 5 x 1 minuto en hielo.

El lisado celular se centrifuga a 13,000 rpm durante 5 minutos y se precipitan 300 µl del sobrenadante en 20 ml de ácido tricloroacético al 10% y DTT (ditiotreitol) 20 mM en acetona fría durante 45 minutos. Las proteínas se recuperan mediante centrifugación y el comprimido se lava tres veces con acetona fría que contiene 20 mM de DTT.

Electroforesis bidimensional en gel

El comprimido de proteína se disuelve en solución para rehidratación de la muestra (urea 7 M, tiourea 2 M, CHAPS al 3% (p/v), azul de bromofenol al 0.002% en agua) y se diluye con la misma solución para enfocamiento isoeléctrico. El enfocamiento isoeléctrico se efectúa utilizando un sistema Zoom IPGRunner (RTM) (Invitrogen Ltd, Carlsbad, CA, E.U.A.) a lo largo de un intervalo de pH no lineal de 3-10 (7 cm) durante un total de 1700 Vh, cargando aproximadamente 15 µg de proteína en cada tira. Antes de la separación en segunda dimensión las tiras se equilibran durante 15 minutos en solución reguladora para equilibrio (Tris-HCl 50 mM, pH 8.8, urea 6 M, 30% de glicerol, 2% (p/v) de SDS, azul de bromofenol al 0.002% en agua) que contiene 65 mM de DTT y después en la misma solución reguladora que contiene 125 mM de yodoacetamida durante otros 15 minutos.

La separación en segunda dimensión se efectúa

utilizando geles NuPage Bis-TrisZoom al 4- 12% y solución reguladora MOPS (Invitrogen Ltd, Carlsbad, CA, EUA). Las proteínas se transfieren mediante blotting a membranas de PVDF Invitrolon (Invitrogen Ltd, Carlsbad, CA, EUA) y se bloquean en una mezcla de 5% de leche descremada en Tween 20 al 0.1% en PBS 10 mM durante 1 hora.

Inmunoabsorción

Para identificar el objetivo del anticuerpo H1L1, la membrana se incuba durante 1 hora con anticuerpo H1L1 purificado en solución de leche descremada al 5% en Tween 20 al 0.1% en PBS 10 mM. Después de lavar, se utiliza un anticuerpo anti-His conjugado con peroxidasa de rábano (Santa Cruz Biotechnologies), diluido a una relación de 1:500 con Tween 20 al 0.1% en PBS 10 mM, para detectar H1L1 unido. Después de lavar con Tween 20 al 0.1% en PBS 10 mM el "blot" se desarrolla utilizando ECL (Amersham biosciences, Little Chalfont, RU).

Para visualizar los objetivos para los anticuerpos de IgG en pacientes infectados con *Clostridium difficile*, las membranas se incuban durante 1 hora con suero diluido 1:20 - 1:50 con Tween 20 al 0.1% en PBS 10 mM. Se utiliza anticuerpo anti-IgG conjugado con peroxidasa de rábano (para ECL) o fosfatasa alcalina (Sigma) en casos en los que la membrana se desarrolla utilizando tabletas de

5-bromo-4-cloro-3-indolil-fosfato/azul nitro tetrazolio SigmaFast (RTM) (Sigma).

Espectrometría de masas por electroaspersión

Las bandas o manchas de proteína cortadas a partir del gel se lavan en bicarbonato de amonio 25 mM durante 10 minutos y se deshidratan utilizando bicarbonato de amonio 25 mM:acetonitrilo 1:2 durante 15 minutos. Después de repetir el lavado con bicarbonato de amonio y el paso de deshidratación, las piezas de gel se secan en un aparato SpeedVac (RTM). Se agregan volúmenes pequeños de tripsina (10 µg/ml en bicarbonato de amonio 25 mM) hasta que las piezas de gel retornan a su tamaño original y se agrega una pequeña cantidad de bicarbonato de amonio 25 mM para mantener húmedas las piezas de gel durante la digestión. Las muestras se digieren durante 4.5 horas a 37°C y los péptidos se extraen durante 15 minutos agregando 80 µl de acetonitrilo. El sobrenadante se seca en un aparato SpeedVac hasta que se evapora todo el acetonitrilo. La muestra se desala utilizando C-18 Zip-Tips (Millipore) y se analizan utilizando espectrometría de masas por tiempo de vuelo con nano-aspersión (Q-TOF, Micromass, Manchester, RU).

Resultados

Aislamiento del objetivo para el anticuerpo H1L1 utilizando geles 2D e inmunoabsorción

Se utilizan geles 2D combinados con inmunoabsorción para ubicar con exactitud el antígeno en el gel 2D. Se comparan dos blots, uno utilizando el suero proveniente del paciente cuyo anticuerpo más abundante es la plantilla para H1L1 y otro utilizando H1L1 expresado y purificado. Ambos reaccionan con la misma mancha de proteína. La misma proteína es antigénica en cuatro pacientes que se han recuperado de diarrea ocasionada por *C. difficile*.

Aislamiento de la proteína objetivo utilizando un método en columna

Se cultiva anaeróbicamente un aislado clínico de *C. difficile* (conocido en la presente invención como cepa CD 14000287, que se aísla a partir de un paciente infectado con *C. difficile*) en caldo de infusión de corazón y cerebro complementado con ácido tioglicólico y clorhidrato de L-cisteína durante 48 horas a 37°C. La suspensión de células se centrifuga en una centrífuga Sorval RC-3B a 5000 rpm durante 20 minutos para comprimir las células y el comprimido de células se lava una vez con PBS y se vuelve a centrifugar como se indicó anteriormente. El comprimido de

células lavado se vuelve a suspender en PBS y se guarda a -20°C hasta uso posterior. Se descongela una alícuota de 2 ml de la suspensión de células congelada y se centrifuga a 14,000 rpm durante 5 minutos en una micro-centrífuga de escritorio para comprimir las células, y las células comprimidas se vuelven a suspender en 4 ml de clorhidrato de guanidina 6 M, pH 8.0. La proteína extraída con guanidina se clarifica mediante centrifugación a 45,000 rpm en una ultra-centrífuga Beckman L8-70M durante una hora a 20°C. El sobrenadante se dializa a temperatura ambiente contra tres veces 2 litros de solución de carbonato de sodio 20 mM, pH 9.5 para intercambiar la proteína extraída en una solución reguladora compatible para la columna AminoLink (vendida por Perbio). Se copulan en forma covalente 2 ml de una solución de anticuerpo H1L1 (~2 mg/ml) en carbonato de sodio 20 mM, pH 9.5, a la resina AminoLink equilibrada con carbonato de sodio 20 mM, pH 9.5, en una columna pre-empacada de 2 ml siguiendo las instrucciones del fabricante (Perbio). El extracto que contiene el antígeno de *C. difficile* (1.5 ml) se aplica después a la columna copulada en forma covalente a H1L1 y se incuba con la resina durante 1 hora a temperatura ambiente. El material no unido se lava a través de la columna con 12 ml de solución reguladora de carbonato de sodio 20 mM, pH 9.5, y el antígeno unido se eluye aplicando

8 ml de solución reguladora de glicina 0.1 M, pH 2.5-3.0. La proteína eluida se neutraliza con Tris 1 M, pH 9.0, antes de concentrar en un adaptador concentrador Amicon de 15 ml hasta 200 µl aproximadamente. Este material de proteína concentrado se analiza después mediante SDS-PAGE al 10% (p/v) y se visualiza utilizando tinción con azul de Coomassie. La banda de proteína que interactúa con H1L1 se identifica después mediante espectrometría de masas.

Identificación de la proteína aislada utilizando espectrometría de masas

La banda o mancha de proteína se corta del gel y se digiere a 37°C con tripsina. Las muestras se analizan utilizando espectroscopia de masas por tiempo de vuelo con nano-electroaspersión, lo cual produce la secuencia de SEQ ID NO: 42.

El mismo péptido se encuentra en las muestras provenientes de los experimentos en gel 2D así como de la proteína que se aísla utilizando el método basado en columna.

La investigación del genoma de *Clostridium difficile* utilizando la búsqueda BLAST en el Instituto Sanger (www.sanger.ac.uk/projects/C_difficile/) suministra SEQ ID NO: 43 como una correspondencia.

Punto isoeléctrico (pI) teórico: 5.74, Peso

molecular: 43430.30 Da que se correlaciona bien con la ubicación de la proteína en el gel 2D en el cual el peso molecular es de aproximadamente 44 kD y pI es de 5.5-6.

Utilizando la secuencia de proteína de SEQ ID NO: 43 en una búsqueda BLAST del NCBI de proteína-proteína en www.ncbi.nlm.nih.gov/BLAST/ utilizando parámetros predeterminados revela similitud elevada con el número de acceso NP_349376 de acetil-CoA-acetiltransferasa (tiolasa) con 68% de identidad a lo largo de 391 aminoácidos.

Clonación de la acetil-CoA acetiltransferasa

Para la clonación y expresión en *E. coli*, la secuencia que codifica para la acetil-CoA acetiltransferasa se amplifica mediante PCR directamente a partir de ADN genómico de *C. difficile*, y se prepara utilizando columnas de centrifugación DNeasy (Qiagen) de conformidad con las instrucciones del fabricante.

Los iniciadores de PCR utilizados se identifican y se diseñan mediante comparación directa con la secuencia de ADN genómico de *C. difficile* que codifica para el par candidato para la acetil-CoA acetiltransferasa, y son SEQ ID NOs: 44 y 45, sintetizadas por SIGMA Genosys.

La amplificación se efectúa utilizando ADN polimerasa *Taq* (Invitrogen) lo que permite la clonación directa independiente de la ligación en el vector de

expresión pBAD-TA (Invitrogen), agregando una marca His_6 fusionada en el extremo C-terminal a la acetil-CoA acetiltransferasa expresada bajo el control del promotor susceptible de inducción por arabinosa araBAD. La mezcla de clonación se transforma en la cepa de expresión TOP10 (Invitrogen) y los recombinantes se identifican utilizando SDS-PAGE e inmunoabsorción utilizando un anticuerpo monoclonal anti-marca His conjugado a peroxidasa (SIGMA). El plásmido resultante es referido en la presente invención como pThioII.

Expresión y purificación de la acetil-CoA acetiltransferasa

Para la sobre-expresión de la proteína de fusión tiolasa-marca His-6, se cultiva *E. coli* cepa TOP10 (pThioII) hasta fase exponencial tardía (OD_{600} 1.0 a 37°C con agitación a 200 rpm) y se induce la expresión de la proteína mediante la adición de arabinosa hasta una concentración final de 0.2%. Después de 240 minutos de crecimiento adicional, las bacterias se recolectan mediante centrifugación (500 g, 10 minutos 4°C) y el comprimido se vuelve a suspender en solución reguladora para lisis (clorhidrato de guanidina 6 M, Tris-HCl 50 mM, pH 8) a 1/20 del volumen de partida y se solubiliza mezclando durante 1 hora a temperatura ambiente. El material insoluble se

elimina mediante un paso adicional de centrifugación (10,000 g, 10 minutos a temperatura ambiente) y el sobrenadante se aplica a una columna Qiagen spin-prep Ni-NTA siguiendo las instrucciones del fabricante. La columna se lava tres veces con 600 µl de solución reguladora para lisis seguido por tres lavados con 600 µl de solución reguladora para lavado 1 (Urea 8 M, Tris/HCl 50 mM, pH 8) y tres veces con 600 µl de solución reguladora para lavado 2 (Urea 8 M, TrisHCl 50 mM, pH 8, imidazol 25 mM). La proteína con marca de His se eluye de la columna con 100 µl de solución reguladora para elución (Urea 8 M, Tris/HCl 50 mM, pH 8, imidazol 250 mM), se repite el paso de elución y se mezclan ambas fracciones. Las muestras se analizan mediante SDS-PAGE.

Evaluación de anticuerpo H1L1 contra acetil-CoA acetiltransferasa clonada

La acetil-CoA acetiltransferasa clonada y purificada se mezcla 1:1 con solución reguladora para carga y DTT y se pone en un bloque de calentamiento a 90°C durante 5 minutos. Se separan 5-15 µl durante 35 minutos en un gel de BisTris al 10% (Invitrogen) utilizando la solución reguladora para corrida NuPAGE-MES SDS (Invitrogen).

Las proteínas se transfieren mediante blotting en

una membrana PVDF Invitrolon (Invitrogen Ltd, Carlsbad, CA, E.U.A.) y se bloquea en una solución de leche descremada al 5% en Tween 20 al 0.1% en PBS 10 mM durante 1 hora. La membrana se incuba con H1L1 a 1:10 (concentración aproximada 400 µg/ml) en Tween 20 al 0.1% en PBS 10 mM durante 1 hora. Se lava 3 veces durante 5 minutos con Tween 20 al 0.1% en PBS 10 mM y después se incuba durante 1 hora con conjugado de proteína S-HRP (Novagen) a 1:1000. El "blot" se desarrolla utilizando tinción Fast DAB de Sigma y muestra una respuesta intensa para la banda de tiolasa localizada justo debajo del marcador de 50 kD.

Por lo tanto, el anticuerpo proveniente de pacientes con infección por *C. difficile* se enfoca contra acetil-CoA acetiltransferasa de *C. difficile* y el anticuerpo sintético construido a partir de las CDR de las cadenas ligera y pesada dominantes de aquellos pacientes es específico contra la acetil-CoA acetiltransferasa de *C. difficile*. Por lo tanto, la acetil-CoA acetiltransferasa de *C. difficile* es un antígeno fuerte y el anticuerpo H1L1 es útil para unirse a éste.

Epítope conservado

Existen tres enzimas consecutivas en la ruta productora de butanol/butirato de *Clostridium acetobutilicum* que también están presentes en el cromosoma

de *Clostridium difficile*. Se ha descubierto que éstas reaccionan con un anti-suero de conejo generado contra las proteínas del sobrenadante proveniente de *Clostridium difficile* (Mullany P et al., FEMS Microbiol Lett., 15 de noviembre de 1994; 124(1):61-7; PMID:8001771).

Suponiendo que las enzimas comparten un epítope conservado, la única región homologa se puede encontrar en el residuo 9-12 en la tiolasa (número de acceso P45362) que está presente en los residuos 5-8 de crotonasa (número de acceso P45361), y esta secuencia conservada es SEQ ID NO: 47.

Concentración Inhibidora Mínima de fármaco de combinación para *Enterococcus faecium* y *Clostridium difficile*

Preparación previa de la prueba

Los organismos se cultivan en una placa de agar sangre Columbia (CBA) para obtener colonias individuales y se incuban a 37°C durante 24 horas (*E. faecium*) o anaeróbicamente durante 48 horas (*C. difficile*).

Los agentes anti-microbianos se preparan de conformidad con la metodología de NCCLS (M7-6A), dando un total de 14 concentraciones para vancomicina y gentamicina y 11 concentraciones para H1L1. Las diluciones iniciales de vancomicina y gentamicina se elaboran en dH_2O, H1L1 se

diluye inicialmente en solución reguladora para formulación (en 100 ml: 3.484 g de arginina (200 mM), 3.006 g de urea (0.5 M), pH 9.5). Se elaboran diluciones subsiguientes en el medio de crecimiento apropiado para el organismo que se está utilizando. Los agentes se guardan en alícuotas a -20°C y se descongelan el día que se utilizan.

Las concentraciones son el doble de la concentración final requerida.

1. Preparación del medio

Medio para *E. faecium*:

Caldo de Mueller Hinton ajustado en cuanto a cationes (NCCLS M7-6A): A 1 litro de caldo de Mueller Hinton (OXOIDE, MHB), se agregan 2 ml de $CaCl_2$ 10 mg/ml y 500 µl de $MgCl_2$ 10 mg/ml

Medio para *C. difficile*:

(1) Caldo de *Brucella* complementado (NCCLS M11-A5): A 900 ml de polvo de caldo para *Brucella* (SIGMA), se agregan 1 ml de Hemina (5 mg/ml) y 1 ml de Vitamina K (1 mg/ml). Después de someter a autoclave se agregan 100 ml de sangre de caballo lisada (5%).

(2) Se prepara medio para *Clostridium* reforzado (RCM) de conformidad con las instrucciones del fabricante: 38 g en 1 litro de dH_2O.

2. Preparación de placa MIC-Tabla 1

Fármaco 1: vancomicina o gentamicina (intervalo de concentración final 0.0625 µg/ml hasta 512 µg/ml).

En una placa de micro-titulación de 96 cavidades con forma de U: se agrega el doble de la concentración requerida de vancomicina o gentamicina (50 µl) de izquierda a derecha a lo largo de la primera hilera de la placa de micro-titulación dejando la cavidad final en blanco. Esto se repite para las otras hileras utilizando concentraciones de vancomicina reducidas dos veces.

Fármaco 2: Anticuerpo H1L1 (intervalo de concentración final (0.25 µg/ml hasta 256 µg/ml)

Se agrega H1L1 (50 ml) hacia abajo en las columnas. Se agrega el doble de la concentración requerida hacia abajo de la primera columna. Esto se repite para las otras columnas moviendo a lo largo de la placa (de izquierda a derecha) concentraciones de H1L1 reducidas dos veces. La última columna se deja en blanco.

La columna final contiene solamente 100 µl de medio de crecimiento.

Las concentraciones continuas de vancomicina y gentamicina se efectúan en la misma manera que la indicada anteriormente pero en una segunda placa de micro-titulación. Las concentraciones de H12L1 son exactamente

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las mismas que las anteriores, al igual que el control con sólo medio en la columna 12.

Preparación del inóculo-suspensión directa de colonia

- Se prepara el inóculo inmediatamente antes de utilizar las bacterias.

- Se elabora una suspensión directa de colonias volviendo a suspender las colonias de una placa de agar de 18 a 24 horas (*E. faecium*) o 48 horas (*C. difficile*) en el medio de crecimiento apropiado o en solución salina estéril.

- Esto se ajusta al estándar de 0.5 de MacFarlands y después se diluye 1:10 en medio de crecimiento (aproximadamente 1 x 10^7 ufc/ml) de conformidad con NCCLS M7-6A (*E. faecium*) y NCCLS M11-A5 (*C. difficile*).

Inoculación de la placa

- Se utilizan 5 µl de la suspensión de inóculo 1:10 preparada como se indicó anteriormente para inocular cada cavidad (inóculo final 5 x 10^4 ufc/ml).

- La placa se inocula desde la cavidad 12 hacia la cavidad 1.

Incubación

- Las placas se incuban a 37°C durante 24 horas (*E. faecium*) o anaeróbicamente durante 48 horas (*C. difficile*).

- Para confirmar el inóculo se diluyen 10 µl del control de crecimiento en 10 ml de solución salina estéril (1:1000) y se siembran 100 µl en una placa CBA y se incuban a 37°C durante 24 horas (*E. faecium*) o anaeróbicamente durante 48 horas (*C. difficile*). Cincuenta colonias son equivalentes a 5×10^4 ufc/ml.

La concentración inhibidora mínima (MIC por sus siglas en inglés) se toma como la concentración más baja de fármaco que inhibe sustancialmente el crecimiento del organismo.

La FIC (concentración inhibidora fraccionaria) se calcula para cada fármaco dividiendo la MIC en presencia del segundo fármaco entre la MIC en ausencia de dicho fármaco. Para cada combinación esto produce dos fracciones, las cuales se suman para producir el FICI (índice de concentración inhibidora fraccionaria): sinergia se define con un valor de ≤ 0.5, indiferencia se define con un valor de >0.5 a <4 y antagonismo se define con un valor de ≥ 4.0.

Resultados

Estos resultados demuestran sinergismo entre H1L1

y vancomicina y gentamicina.

Conclusiones

Estos resultados demuestran sinergismo tanto entre (i) H1L1 y vancomicina, como entre (ii) H1L1 y gentamicina contra *C. difficile* 14000287 y *C. difficile* NCTC 11204. Estos muestran sinergismo entre vancomicina y H1L1 en *Enterococcus faecium* resistente a vancomicina.

Crecimiento de *Clostridium difficile* en presencia de concentraciones variables de ácidos grasos de cadena corta (SCFA) y anticuerpo H1L1

Preparación de SCFA

Los tres SCFA utilizados son acetato, propionato y butirato.

Cada uno se prepara de la siguiente manera hasta una concentración 1 M:

Acetato de sodio Peso fórmula 82.03: 82.03 g en 1 litro de dH_2O

Butirato de sodio Peso fórmula 110.09: 110.09 g en 1 litro de dH_2O

Propionato de sodio Peso fórmula 96.06: 96.06 g en 1 litro de dH_2O.

Las tres soluciones se combinan en una relación

de 70:20:10 respectivamente. Por lo tanto, en la solución final, las concentraciones son acetato de sodio 0.7 M, butirato de sodio 0.2 M y propionato de sodio 0.1 M. La concentración final de los SCFA es 1 M.

A partir de esta solución de reserva se preparan concentraciones de 5, 10, 20, 30, 40 y 50 mM de SCFA totales utilizando medio para *Clostridium* reforzado (RCM, DIFCO).

Preparación del medio

Se prepara medio para *Clostridium* reforzado (RCM) de conformidad con las instrucciones del fabricante: 38 g en 1 litro de dH_2O.

Curva de crecimiento

Día antes de la prueba

Los cultivos solamente se retiran de las condiciones anaeróbicas inmediatamente antes del uso. La exposición a condiciones aeróbicas no es mayor de 30 minutos.

Se prepara una suspensión nocturna de *C. difficile* inoculando 10 ml de medio RCM con colonias provenientes de una placa de agar sangre Columbia de 48 horas de edad del organismo. La suspensión se incuba durante 24 horas a 37°C bajo condiciones anaeróbicas.

100 F

Día de la prueba

Placa de control

En una placa de micro-titulación de 96 cavidades de fondo plano se colocan en la cavidad las soluciones de control (200 µl) como se indica más adelante en la tabla 4. Cada control utiliza tres cavidades.

Placa de prueba

En una placa de micro-titulación de 96 cavidades de fondo plano (hileras marcadas A-H, las columnas se marcan como 1-12) se colocan en las cavidades las soluciones de prueba (100 µl) como se describe con mayor detalle más adelante. Las dos soluciones de prueba, SCFA más H1L1, producen un volumen combinado de 200 µl. Cada prueba utiliza dos cavidades. Las cavidades en la hilera A contienen 50 mM de SCFA; en la hilera B, 40 mM de SCFA; en la hilera C, 30 mM de SCFA; en la hilera D, 20 mM de SCFA; en la hilera E, 10 mM de SCFA; en la hilera F, 0.5 mM de SCFA. Las cavidades en las columnas 1 y 2 contienen 16 µg/ml de H1L1; en las columnas 3 y 4, 8 µg/ml de H1L1; en las columnas 5 y 6, 4 µg/ml de H1L1; en las columnas 7 y 8, 2 µg/ml de H1L1; en las columnas 9 y 10, 1 µg/ml de H1L1; en las columnas 11 y 12, 0.5 µg/ml de H1L1. La hilera G está vacía. Se colocan 200 µl de RCM en todas las cavidades en la hilera H como un control de crecimiento

para la placa.

Preparación del inóculo

Los cultivos solamente se retiran de las condiciones anaeróbicas inmediatamente antes del uso. La exposición a condiciones aeróbicas no es mayor de 30 minutos.

La suspensión nocturna, cultivo en fase logarítmica tardía, se utiliza para inocular las placas de micro-titulación. Se agregan 20 µl (aproximadamente 10% de inóculo) a todas las cavidades, excepto para el control de medio/negativo en la placa de control.

Las placas se incuban a 37°C bajo condiciones anaeróbicas durante 24 horas.

Se efectúan lecturas de densidad óptica (DO_{600} nm) en los puntos de tiempo 0 y 24 horas. Se determinan lecturas de DO_{600} nm promedio.

Resultados

Controles-véase tabla 5

Conclusiones

1. Sin efecto de 10, 20, 30, y 40 mM de SCFA por sí mismos, pero cierta cantidad de decremento con 50 mM de SCFA.

2. Sin efecto con 0.5, 1 , 2, 4, y 8 µg/ml de H1L1 pero inhibición con 16 µg/ml de H1L1.

Pruebas

Véase tabla 6.

Conclusiones

Indiferencia con 1, 2 y 4 µg/ml de H1L1 y 5 mM de SCFA (datos no mostrados). Sinergismo con 8 µg/ml de H1L1 y SCFA de 10-50 mM con densidad óptica a las 24 horas que cae hasta un intervalo de 0.296-0.330. El efecto es más evidente con 16 µg/ml de H1L1 y 50 mM de SCFA.

TABLA 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	Sólo medio
B	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	Sólo medio
C	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	Sólo medio
D	D1	D3	D4	D4	D5	D6	D7	D8	D9	D10	D11	Sólo medio
E	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	Sólo medio
F	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	Sólo medio
G	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	Sólo medio
H	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	Sólo medio

Las concentraciones de fármaco en esta etapa son la mitad de la concentración que se coloca en las cavidades (50 µl + 50 µl = dilución 1:2)

A, B, etc. = vancomicina o gentamicina

1, 2, 3, etc = + H1L1

TABLA 2

Especie	Agente	MIC (µg/ml) de cada agente		FIC (µg/ml)	FICI	Resultado
		Sólo	Combinación			
Clostridium difficile NCR 1104 (caldo para Brucella)	Vancomicina	1	0.125	0.125	0.125	Sinergia
	H1L1	512	0.25	0.0004		
Enterococcus faecium	Vancomicina	128	16	0.12	0.37	Sinergia
	H1L1	512	128	0.25		

TABLA 3

Especies	Agente	MIC (µg/ml) de cada agente		FIC (µg/ml)	FICI	Resultado
		Sólo	Combinación			
C. difficile 14000287 (caldo para Brucella)	Gentamicina	8	2	0.25	0.266	Sinergia
	H1L1	512	8	0.0156		
C. difficile NCTC 11204 (RCM)	Gentamicina	16	4	0.25	0.375	Sinergia
	H1L1	512	64	0.125		

TABLA 4

	1	2	3	4	5	6	7	8	9	10	11	12
A	RCM sin inóculo			RCM + inóculo								
B	RCM + 50 mM de SCFA			RCM + 40 mM de SCFA			RCM + 30 mM de SCFA			RCM + 20 mM de SCFA		
C	RCM + 10 mM de SCFA			RCM + 5 mM de SCFA								
D	RCM + 16 µg/ml de H1L1			RCM + 8 µg/ml de H1L1			RCM + 4 µg/ml de H1L1			RCM + 2 µg/ml de H1L1		
E	RCM + 1 µg/ml de H1L1			RCM + 0.5 µg/ml de H1L1								
F												
G												
H												

TABLA 5

No. de experimento	H1L1 (µg/ml)	SCFA (mM)	Densidad óptica promedio a las 24 horas
1	-	-	0.452
2	-	5	0.460
3	-	10	0.429
4	-	20	0.452
5	-	30	0.480
6	-	40	0.440
7	-	50	0.370

TABLA 5

No. de experimento	H1L1 (µg/ml)	SCFA (mM)	Densidad óptica promedio a las 24 horas
8	0.5	-	0.450
9	1	-	0.440
10	2	-	0.440
11	4	-	0.440
12	8	-	0.420
13	16	-	0.334

TABLA 6

No. de experimento	H1L1 (µg/ml)	SCFA (mM)	Densidad óptica promedio a las 24 horas
1	8	10	0.310
2	16	10	0.308
3	8	20	0.296
4	16	20	0.228
5	8	30	0.305
6	16	30	0.308
7	8	40	0.310
8	16	40	0.303
9	8	50	0.330
10	16	50	0.281

NOVEDAD DE LA INVENCION

Habiendo descrito el presente invento se considera como novedad y por lo tanto se reclama como propiedad lo contenido en las siguientes:

REIVINDICACIONES

1.- El uso de un inhibidor de acetil-CoA acetiltransferasa en la fabricación de un medicamento para el tratamiento o profilaxis de infección por *Clostridium difficile*.

2.- El uso de conformidad con la reivindicación 1, caracterizado porque dicha acetil-CoA acetiltransferasa proviene de *Clostridium difficile*.

3.- El uso de conformidad con cualquiera de las reivindicaciones precedentes, caracterizado porque dicho inhibidor es un anticuerpo o un fragmento de unión a antígeno del mismo.

4.- El uso de conformidad con la reivindicación 3, caracterizado porque dicho anticuerpo o fragmento de unión a antígeno del mismo es específico contra un epítope desplegado por el péptido que tiene la secuencia de SEQ ID NO: 47.

5.- El uso de conformidad con cualquiera de las

reivindicaciones 3 ó 4, caracterizado porque dicho anticuerpo o fragmento de unión a antígeno del mismo tiene 1-3 regiones determinantes de complementariedad (CDRs) de cadena VH que tienen las secuencias de SEQ ID NOs:2-4 respectivamente.

6.- El uso de conformidad con la reivindicación 5, caracterizado porque dicha cadena VH del anticuerpo o fragmento de unión a antígeno del mismo tiene la secuencia de SEQ ID NO: 1.

7.- El uso de conformidad con cualquiera de las reivindicaciones 3-6, caracterizado porque dicho anticuerpo o fragmento de unión a antígeno del mismo tiene 1-3 regiones determinantes de complementariedad (CDRs) de cadena VL que tienen las secuencias de SEQ ID NOs: 17-19.

8.- El uso de conformidad con la reivindicación 7, caracterizado porque dicha cadena VL del anticuerpo o fragmento de unión a antígeno del mismo tiene la secuencia SEQ ID NO: 16.

9.- El uso de conformidad con la reivindicación 3, caracterizado porque dicho anticuerpo o fragmento de unión a antígeno del mismo tiene la secuencia de SEQ ID NO:41.

10.- El uso de conformidad con cualquiera de las reivindicaciones precedentes, caracterizado porque el

medicamento puede comprender de manera adicional por lo menos uno del grupo de antibióticos que consiste de: gentamicina, vancomicina y metronidazol.

11.- Una preparación combinada para el tratamiento de infección por *Clostridium difficile*, que comprende:

(i) un inhibidor de acetil-CoA acetiltransferasa; y

(ii) por lo menos uno del grupo de antibióticos que consiste de: gentamicina, vancomicina, y metronidazol.

12.- Un anticuerpo aislado y/o purificado o fragmento de unión a antígeno del mismo que comprende por lo menos uno del grupo que consiste de:

(i) 1-3 regiones determinantes de complementariedad (CDRs) de cadena VH que tengan las secuencias de SEQ ID NOs:2-4 respectivamente; y

(ii) 3 regiones determinantes de complementariedad (CDRs) de cadena VL que tengan la secuencia de SEQ ID NOs:19.

13.- El anticuerpo aislado y/o purificado o fragmento de unión a antígeno del mismo de conformidad con la reivindicación 12, caracterizado porque dicha cadena VH tiene la secuencia de SEQ ID NO:1.

14.- El anticuerpo aislado y/o purificado o fragmento de unión a antígeno del mismo de conformidad con cualquiera de las reivindicaciones 12 ó 13, caracterizado

porque dicha cadena VL tiene la secuencia de SEQ ID NO: 16.

15.- El anticuerpo aislado y/o purificado o fragmento de unión a antígeno del mismo de conformidad con la reivindicación 12, caracterizado porque dicho anticuerpo tiene la secuencia de SEQ ID NO:41.

16.- Una molécula de ácido nucleico que codifica para un anticuerpo o fragmento de unión a antígeno del mismo de conformidad con la reivindicación 15.

17.- Una molécula de ácido nucleico de conformidad con la reivindicación 16, que tiene la secuencia de SEQ ID NO:46.

18.- El uso de un inhibidor de acetil-CoA acetiltransferasa y vancomicina en la fabricación de un medicamento para la profilaxis o tratamiento de infección por *Enterococcus faecium* y *Enterococcus faecalis*.

19.- El uso de conformidad con la reivindicación 8, caracterizado porque dicho *Enterococcus faecium* o *Enterococcus faecalis* es resistente a vancomicina.

20.- El uso de conformidad con cualquiera de las reivindicaciones 18 ó 19, caracterizado porque dicha acetil-CoA acetiltransferasa proviene de *Clostridium difficile*.

21.- El uso de conformidad con cualquiera de las reivindicaciones 18-20, caracterizado porque dicho inhibidor es un anticuerpo o un fragmento de unión a

antígeno del mismo.

22.- El uso de conformidad con la reivindicación 21, caracterizado porque dicho anticuerpo o fragmento de unión a antígeno del mismo es específico contra un epítope desplegado por el péptido que tiene la secuencia de SEQ ID NO: 47.

23.- El uso de conformidad con cualquiera de las reivindicaciones 21 ó 22, caracterizado porque dicho anticuerpo o fragmento de unión a antígeno del mismo tiene 1-3 regiones determinantes de complementariedad (CDRs) de cadena VH que tienen las secuencias de SEQ ID NOs: 2-4 respectivamente.

24.- El uso de conformidad con la reivindicación 23, caracterizado porque dicha cadena VH del anticuerpo o fragmento de unión a antígeno del mismo tiene la secuencia de SEQ ID NO:1.

25.- El uso de conformidad con cualquiera de las reivindicaciones 21-24, caracterizado porque dicho anticuerpo o fragmento de unión a antígeno del mismo tiene 3 regiones determinantes de complementariedad (CDRs) de cadena VL que tienen la secuencia de SEQ ID NOs:19.

26.- El uso de conformidad con la reivindicación 25, caracterizado porque dicha cadena VL del anticuerpo o fragmento de unión a antígeno del mismo tiene la secuencia

de SEQ ID NO: 16.

27.- El uso de conformidad con la reivindicación 21, caracterizado porque dicho anticuerpo o fragmento de unión a antígeno del mismo tiene la secuencia de SEQ ID NO: 41.

28.- Una preparación combinada para el tratamiento de infección por *Enterococcus faecium* o *Enterococcus faecalis*, que comprende:

(i) un inhibidor de acetil-CoA acetiltransferasa; y

(ii) vancomicina.

29.- Un método de tratamiento de infección por *Clostridium difficile*, que comprende administrar una cantidad terapéuticamente efectiva de un inhibidor de acetil-CoA acetiltransferasa a un paciente en necesidad del mismo.

30.- Un método de conformidad con la reivindicación 29, que comprende adicionalmente administrar una cantidad terapéuticamente efectiva de por lo menos uno del grupo de antibióticos que consiste de: gentamicina, vancomicina y metronidazol.

31.- Un método de tratamiento de infección por *Enterococcus faecium* o *Enterococcus faecalis*, que comprende administrar una cantidad terapéuticamente efectiva de:

(i) un inhibidor de acetil-CoA acetiltransferasa; y

(ii) vancomicina

a un paciente en necesidad del mismo.

32.- Un método de conformidad con la reivindicación 31, caracterizado porque dicho *Enterococcus faecium* o *Enterococcus faecalis* es resistente a vancomicina.

RESUMEN DE LA INVENCION

La presente invención se refiere a compuestos, medicamentos y tratamientos para infección por *Clostridium difficile*, junto con anticuerpos aislados novedosos y su uso en la misma. La presente invención también se refiere a la profilaxis y tratamiento de infección por *E. faecium* y *E. faecalis* y provee medicamentos y tratamientos para las mismas.

LISTADO DE SECUENCIAS

<110> Neutec Pharma Plc
Burnie, James
Matthews, Ruth
Carter, Tracey

<120> Tratamiento de Infecciones Bacterianas

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PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

(Chapter II of the Patent Cooperation Treaty)

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference WAMP101123-WO	FOR FURTHER ACTION	See Form PCT/IPEA/416
International application No. PCT/GB2005/002607	International filing date (day/month/year) 01.07.2005	Priority date (day/month/year) 02.07.2004
International Patent Classification (IPC) or national classification and IPC INV. C07K16/12		

Applicant
NEUTEC PHARMA PLC et al.

1. This report is the international preliminary examination report, established by this International Preliminary Examining Authority under Article 35 and transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 8 sheets, including this cover sheet.
3. This report is also accompanied by ANNEXES, comprising:
   a. ☒ *sent to the applicant and to the International Bureau)* a total of 2 sheets, as follows:
      ☒ sheets of the description, claims and/or drawings which have been amended and are the basis of this report and/or sheets containing rectifications authorized by this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions).
      ☐ sheets which supersede earlier sheets, but which this Authority considers contain an amendment that goes beyond the disclosure in the International application as filed, as indicated in item 4 of Box No. I and the Supplemental Box.
   b. ☐ *(sent to the International Bureau only)* a total of (indicate type and number of electronic carrier(s)) , containing a sequence listing and/or tables related thereto, in electronic form only, as indicated in the Supplemental Box Relating to Sequence Listing (see Section 802 of the Administrative Instructions).
4. This report contains indications relating to the following items:
   - ☒ Box No. I Basis of the report
   - ☒ Box No. II Priority
   - ☒ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - ☐ Box No. IV Lack of unity of invention
   - ☒ Box No. V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - ☐ Box No. VI Certain documents cited
   - ☐ Box No. VII Certain defects in the international application
   - ☐ Box No. VIII Certain observations on the international application

Date of submission of the demand	Date of completion of this report
12.04.2006	28.06.2006

Name and mailing address of the international preliminary examining authority:
European Patent Office
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Authorized officer
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Box No. I Basis of the report

1. With regard to the language, this report is based on

   ☒ the international application in the language in which it was filed

   ☐ a translation of the international application into , which is the language of a translation furnished for the purposes of:
   - ☐ international search (under Rules 12.3(a) and 23.1(b))
   - ☐ publication of the international application (under Rule 12.4(a))
   - ☐ international preliminary examination (under Rules 55.2(a) and/or 55.3(a))

2. With regard to the elements* of the international application, this report is based on *(replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report)*:

Description, Pages

1-33	as originally filed

Sequence listings part of the description, Pages

1-14	as originally filed

Claims, Numbers

1-8, 15-22, 30-32	as originally filed
9-14, 23-29	received on 12.04.2006 with letter of 11.04.2006

   ☒ a sequence listing and/or any related table(s) - see Supplemental Box Relating to Sequence Listing

3. ☐ The amendments have resulted in the cancellation of:
   - ☐ the description, pages
   - ☐ the claims, Nos.
   - ☐ the drawings, sheets/figs
   - ☐ the sequence listing *(specify)*:
   - ☐ any table(s) related to sequence listing *(specify)*:

4. ☐ This report has been established as if (some of) the amendments annexed to this report and listed below had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).
   - ☐ the description, pages
   - ☐ the claims, Nos.
   - ☐ the drawings, sheets/figs
   - ☐ the sequence listing *(specify)*:
   - ☐ any table(s) related to sequence listing *(specify)*:

* If item 4 applies, some or all of these sheets may be marked "superseded."

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INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

International application No.
PCT/GB2005/002607

Box No. II Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:
   ☐ copy of the earlier application whose priority has been claimed (Rule 66.7(a)).
   ☐ translation of the earlier application whose priority has been claimed (Rule 66.7(b)).

2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid (Rule 64.1). Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:

   see separate sheet

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

International application No. PCT/GB2005/002607

Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application,

☒ claims Nos. 1,2,10,11,18-20,28-32

because:

☒ the said international application, or the said claims Nos. 29-32 relate to the following subject matter which does not require an international preliminary examination *(specify)*:

see separate sheet

☐ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. are so unclear that no meaningful opinion could be formed *(specify)*:

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed *(specify)*.

☒ no international search report has been established for the said claims Nos. 1,2,10,11,18-20,28-32

☐ a meaningful opinion could not be formed without the sequence listing: the applicant did not, within the prescribed time limit:

☐ furnish a sequence listing on paper complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Preliminary Examining Authority in a form and manner acceptable to it.

☐ furnish a sequence listing in electronic form complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Preliminary Examining Authority in a form and manner acceptable to it.

☐ pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rules 13*ter*.1(a) or (b) and 13*ter*.2.

☐ a meaningful opinion could not be formed without the tables related to the sequence listings; the applicant did not, within the prescribed time limit, furnish such tables in electronic form complying with the technical requirements provided for in Annex C-*bis* of the Administrative Instructions, and such tables were not available to the International Preliminary Examining Authority in a form and manner acceptable to it.

☐ the tables related to the nucleotide and/or amino acid sequence listing, if in electronic form only, do not comply with the technical requirements provided for in Annex C-*bis* of the Administrative Instructions.

☐ See separate sheet for further details

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

International application No.
PCT/GB2005/002607

Box No. V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-32
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-11, 18-32
	No:	Claims	12-17
Industrial applicability (IA)	Yes:	Claims	1-28
	No:	Claims	

2. Citations and explanations (Rule 70.7):

see separate sheet

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

International application No.
PCT/GB2005/002607

Supplemental Box relating to Sequence Listing

Continuation of Box I, item 2:

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, this report was established on the basis of:

   a. type of material:

   ☒ a sequence listing

   ☐ table(s) related to the sequence listing

   b. format of material:

   ☒ on paper

   ☒ in electronic form

   c. time of filing/furnishing:

   ☒ contained in the international application as filed

   ☒ filed together with the international application in electronic form

   ☐ furnished subsequently to this Authority for the purposes of search and/or examination

   ☐ received by this Authority as an amendment* on

2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table(s) relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

* *If item 4 in Box No. I applies, the listing and/or table(s) related thereto, which form part of the basis of the report, may be marked "superseded."*

Re Item II:

The validity of the priority claim has not been considered because the International Searching Authority does not have in its possession a copy of the earlier application whose priority has been claimed or, where required, a translation of said earlier application. This report has nevertheless been established on the assumption that the relevant date is the claimed priority date.

Re Item III:

1. The present report is only formulated with respect to those parts of the claims for which an international search report has been established, i.e. parts relating to those inhibitors of acetyl-CoA acetyltransferase which are antibodies or antigen binding fragments thereof.

2. Claims 29-32 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. In this respect the following should be noted: For the assessment of these claims on the question whether they are industrially applicable, no unified criteria exist in PCT. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject matter of claims to the use of a compound in medical treatment, but will allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.
Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(I) PCT).

Re Item V:

1. Novelty:

The subject matter of claims 1-32 complies with Articles 33(2) PCT.

2. Inventive step:

The subject-matter of claims 12-17 relates to specific antibodies, which antibodies apparently are reactive with *Clostridium* and *Enterococcus* acetyl-CoA acetyltransferase. It is noted that said claims are product claims which do not refer to any therapeutic application. The closest prior art to assess the inventiveness of said claims is any of documents D8 (Winzer) and D9 (Sliwkowski), disclosing the acetyl-CoA acetyltransferase of *Clostridium*. Once an enzyme is known, no inventive skill is needed to provide antibodies against said enzyme. Therefore the subject matter of claims 12-17 is not considered inventive. It seems that the inventive concept of the present application is the surprising fact that antibodies against acetyl-CoA acetyltransferase (not a surface antigen) can be used for the treatment of *Clostridium* and *Enterococcus* infection. Therefore inventive step is recognized for therapeutic applications of said antibodies, as defined in claims 1-11 and 18-32.

9. The use according to claim 3, said antibody or antigen binding fragment thereof having the sequence of SEQ ID NO: 41.

10. The use according to any of the preceding claims, the medicament additionally comprising at least one of the group of antibiotics consisting: gentamicin, vancomycin and metronidazole.

11. A combined preparation for the treatment of infection by *Clostridium difficile*, comprising:

(i) an inhibitor of acetyl-CoA acetyltransferase; and

(ii) at least one of the group of antibiotics consisting: gentamicin, vancomycin and metronidazole.

12. Isolated and/or purified antibody or antigen binding fragment thereof comprising at least one of the group consisting of:

(i) VH chain complementarity determining regions (CDRs) 1-3 having the sequences of SEQ ID NOs: 2-4 respectively; and

(ii) VL chain complementarity determining regions (CDR) 3 having the sequence of SEQ. ID NO: 10.

13. Isolated and/or purified antibody or antigen binding fragment thereof according to claim 12, said VH chain having the sequence of SEQ ID NO: 1.

14. Isolated and/or purified antibody or antigen binding fragment thereof according to either of claims 12 or 13, said VL chain having the sequence of SEQ ID NO: 16.

23. The use according to either of claims 21 or 22, said antibody or antigen binding fragment thereof having VH chain complementarity determining regions (CDRs) 1-3 having the sequences of SEQ ID NOs: 2-4 respectively.

24. The use according to claim 23, said antibody or antigen binding fragment thereof VH chain having the sequence of SEQ ID NO: 1.

25. The use according to any of claims 21-24, said antibody or antigen binding fragment thereof having VL chain complementarity determining regions (CDR) 3 having the sequence of SEQ. ID NO: 19.

26. The use according to claim 25, said antibody or antigen binding fragment thereof VL chain having the sequence of SEQ ID NO: 16.

27. The use according to claim 21, said antibody or antigen binding fragment thereof having the sequence of SEQ ID NO: 41.

28. A combined preparation for the treatment of infection by *Enterococcus faecium* or *Enterococcus faecalis*, comprising:

(i) an inhibitor of acetyl-CoA acetyltransferase; and

(ii) vancomycin.

29. A method of treatment of infection by *Clostridium difficile*, comprising administering a therapeutically effective quantity of an inhibitor of acetyl-CoA acetyltransferase to a patient in need of same.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
WA/MP101123-WO		
International application No.	International filing date (day/month/year)	(Earliest) Priority Date (day/month/year)
PCT/GB2005/002607	01/07/2005	02/07/2004
Applicant		
NEUTEC PHARMA PLC		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 7 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report
   a. With regard to the language, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.
      [ ] The international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).
   b. [X] With regard to any nucleotide and/or amino acid sequence disclosed in the international application, see Box No. I.

2. [X] Certain claims were found unsearchable (See Box II).

3. [ ] Unity of invention is lacking (see Box III).

4. With regard to the title,
   [X] the text is approved as submitted by the applicant.
   [ ] the text has been established by this Authority to read as follows:

5. With regard to the abstract,
   [X] the text is approved as submitted by the applicant.
   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. With regard to the drawings,
   a. the figure of the drawings to be published with the abstract is Figure No. _____
      [ ] as suggested by the applicant.
      [ ] as selected by this Authority, because the applicant failed to suggest a figure.
      [ ] as selected by this Authority, because this figure better characterizes the invention.
   b. [ ] none of the figures is to be published with the abstract.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2005/002607

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. type of material

   - [x] a sequence listing
   - [ ] table(s) related to the sequence listing

   b. format of material

   - [x] in written format
   - [x] in computer readable form

   c. time of filing/furnishing

   - [x] contained in the international application as filed
   - [x] filed together with the international application in computer readable form
   - [ ] furnished subsequently to this Authority for the purpose of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International Application No
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K16/12 A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, EMBASE, Sequence Search, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 2004/094474 A (NEUTEC PHARMA PLC; BURNIE, JAMES, PETER; MATTHEWS, RUTH, CHRISTINE) 4 November 2004 (2004-11-04) see especially SEQ ID NO:s 27 and 32; the whole document	12-17
X	WO 01/27279 A (CAMBRIDGE ANTIBODY TECHNOLOGY; EDWARDS, BRYAN, MICHAEL; MAIN, SARAH, H) 19 April 2001 (2001-04-19) see especially SEQ ID NO: 62 the whole document	12-17
X	WO 01/44300 A (CAMBRIDGE ANTIBODY TECHNOLOGY LIMITED; WEBSTER, CARL; OSBOURN, JANE; W) 21 June 2001 (2001-06-21) see especially SEQ ID NO: 76; the whole document	12-17

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 August 2005	20/09/2005

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Giebeler, K

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Form PCT/ISA/210 (second sheet) (January 2004)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01/96599 A (PHILOGEN S.R.L; CASTELLANI, PATRIZIA; ZARDI, LUCIANO; ZIJLSTRA, ANDRIE) 20 December 2001 (2001-12-20) see especially SEQ ID NO:4; the whole document	12-17
X	WO 03/048321 A (ALEXION PHARMACEUTICALS, INC; ROTHER, RUSSELL; WU, DAYANG) 12 June 2003 (2003-06-12) see espcially SEQ ID NO: 106; the whole document	12-17
A	WO 03/052416 A (NEUTEC PHARMA PLC; BURNIE, JAMES, PETER; MATTHEWS, RUTH, CHRISTINE; RI) 26 June 2003 (2003-06-26) cited in the application the whole document	1-32
A	WO 02/062379 A (THE PROVOST, FELLOWS AND SCHOLARS OF THE COLLEGE OF THE HOLY AND UNIDI) 15 August 2002 (2002-08-15) cited in the application the whole document	1-32
A	WINZER KLAUS ET AL: "Differential regulation of two thiolase genes from Clostridium acetobutylicum DSM 792" JOURNAL OF MOLECULAR MICROBIOLOGY AND BIOTECHNOLOGY, vol. 2, no. 4, October 2000 (2000-10), pages 531-541, XP009053009 ISSN: 1464-1801 the whole document	1-32
A	SLIWKOWSKI M X ET AL: "INCORPORATION AND DISTRIBUTION OF SELENIUM INTO THIOLASE FROM CLOSTRIDIUM-KLUYVERI" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 260, no. 5, 1985, pages 3140-3144, XP002342751 ISSN: 0021-9258 the whole document	1-32

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.1

Although claims 29-32 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box II.2

Claims Nos.: -

Present claims 1,2,10,11,18-20, and 28-32 relate to the use of a compound defined by reference to a desirable characteristic or property, namely to an inhibitor of acetyl-CoA acetyltransferase.
The claims cover all compounds having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and disclosure within the meaning of Article 5 PCT for only a very limited number of such compounds. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the compound by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to those inhibitors of acetyl-CoA acetyltransferase which are antibodies or antigen binding fragments thereof.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.5), should the problems which led to the Article 17(2) declaration be overcome.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2005/002607

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Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 29-32 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB2005/002607

144/43

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2004094474 A	04-11-2004	WO 2004094474 A1	04-11-2004
WO 0127279 A	19-04-2001	AU 7676300 A	23-04-2001
		WO 0127279 A1	19-04-2001
WO 0144300 A	21-06-2001	AU 770115 B2	12-02-2004
		AU 1713901 A	25-06-2001
		CA 2393292 A1	21-06-2001
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		JP 2003516745 T	20-05-2003
WO 0196599 A	20-12-2001	AU 8182401 A	24-12-2001
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WO 03048321 A	12-06-2003	AU 2002359568 A1	17-06-2003
		CA 2468744 A1	12-06-2003
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		JP 2005511706 T	28-04-2005
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		US 2003219861 A1	27-11-2003
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		AU 2002352394 A1	30-06-2003
		CA 2471570 A1	26-06-2003
		DE 60202877 D1	10-03-2005
		EP 1415002 A2	06-05-2004
		ES 2236605 T3	16-07-2005
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		IE 20020097 A1	28-05-2003
		US 2003054009 A1	20-03-2003

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
T/GB2004/001619

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
DE 19739685	A	11-03-1999	DE	19739685 A1	11-03-1999
			AT	254139 T	15-11-2003
			AU	9742698 A	29-03-1999
			BR	9815367 A	06-11-2001
			CA	2303202 A1	18-03-1999
			CN	1273588 T	15-11-2000
			DE	59810172 D1	18-12-2003
			WO	9912971 A2	18-03-1999
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			ES	2210832 T3	01-07-2004
			JP	2001515920 T	25-09-2001
			US	6667035 B1	23-12-2003
			US	2004137601 A1	15-07-2004
WO 0012562	A	09-03-2000	AT	217889 T	15-06-2002
			AU	766551 B2	16-10-2003
			AU	5692399 A	21-03-2000
			BR	9913435 A	25-09-2001
			CA	2341276 A1	09-03-2000
			CN	1317015 T	10-10-2001
			DE	69901569 D1	27-06-2002
			DE	69901569 T2	19-12-2002
			DK	1107996 T3	16-09-2002
			EP	1107996 A1	20-06-2001
			ES	2177316 T3	01-12-2002
			JP	2002523081 T	30-07-2002
			NZ	509430 A	26-04-2002
			PT	1107996 T	31-10-2002
			WO	0012562 A1	09-03-2000
			US	6624295 B1	23-09-2003
			ZA	200100681 A	24-01-2002
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			AU	703319 B2	25-03-1999
			AU	1103697 A	27-06-1997
			CA	2239519 A1	12-06-1997
			DE	69612509 D1	17-05-2001
			DE	69612509 T2	18-10-2001
			DK	865492 T3	11-06-2001
			EP	0865492 A1	23-09-1998
			ES	2157473 T3	16-08-2001
			WO	9720932 A1	12-06-1997
			JP	2000504204 T	11-04-2000
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			EP	1415002 A2	06-05-2004
			WO	03052416 A2	26-06-2003

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: Part of claims 1-17 and 20-37

The scope of part of present claims 1-17 and 20-37 relate to an extremely large number of possible compounds and methods. In fact, claim 1 and all claims related thereto contain so many options amongst the cited claimed amino acid sequences and possible combinations thereof, that a lack of clarity and conciseness within the meaning of Article 6 PCT arises to such an extent as to render a meaningful search of the claims impossible. Consequently, the search for the first invention has been carried out for those parts of the application which could be considered to be clear and concise, namely a panel of 10 "binding members" which each contain one of the VH-CDR sequences shown in Table 4, but restricted to those VH-CDRs of the "FATs" indicated in claim 18, as well as the corresponding ten single binding members (claim 26 onwards) with the distinct VH variable domains as indicated in claim 19 (bringing the present search up to 20 distinct sequence searches in the relevant databases, namely 10 CDR3 sequences (see claim 18) and in addition the corresponding antibody VH variable domains (see claim 19)). In this context it should be noted that the present application as filed does not appear to highlight the relevance of any further combination of 10 binding partners which might be considered essential/relevant to carry out the invention. In this respect, it should also be noted that the relevance of the chosen combination as claimed in claims 18 and 19 is per se not ad hoc apparent from the description and examples of the application as filed, but was chosen as they appeared as the only specific combination of least ten binding members in the present claimed subject-matter.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 00/03900

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5631009 A	20-05-1997	AT 175422 T	15-01-1999
		AU 664147 B	02-11-1995
		AU 2568692 A	27-04-1993
		CA 2116033 A	01-04-1993
		DE 69228121 D	18-02-1999
		DE 69228121 T	10-06-1999
		DK 597043 T	30-08-1999
		EP 0597043 A	18-05-1994
		WO 9306131 A	01-04-1993
		GB 2259706 A,B	24-03-1993
		JP 6510780 T	01-12-1994
		NZ 244349 A	26-10-1994
US 5102658 A	07-04-1992	CA 1302319 A	02-06-1992

Form PCT/ISA/210 (patent family annex) (July 1992)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: (9, 11, 21, 42, 44) - complete; (1-8, 19, 20, 23-41, 52-64) - partial

Clostridium difficile S layer protein (SlpA) comprising SEQ ID NO:1 and its corresponding gene (slpA), which comprises SEQ ID NO:3; epitopes, homologs, derivatives, variants or fragments thereof. Chimeras comprising any of the previously mentioned polynucleotides/(poly)peptides. Antibodies against those (poly)peptides. Vaccines comprising any of the former and methods for prophylaxis/treatment of C. difficile-associated diseases based on the use thereof.

2. Claims: (10, 13, 14, 17, 18, 22, 43, 46, 47, 50, 51) - complete; (1-8, 19, 20, 23-41, 52-64) - partial

Idem as subject 1, but restricted to a gene comprising SEQ ID NOs: 5, 6, 9 or 10 and a polypeptide/peptide comprising SEQ ID NO:2.

3. Claims: (12, 45) - complete; (1-8, 19, 20, 23-37, 39-41, 52-54, 56-64) - partial

Idem as subject 1, but restricted to a gene comprising SEQ ID NO:4.

4. Claims: (15, 48) - complete; (1-8, 19, 20, 23-37, 39-41, 52-54, 56-64) - partial

Idem as subject 1, but restricted to a gene comprising SEQ ID NO:7.

5. Claims: (16, 49) - complete; (1-8, 19, 20, 23-37, 39-41, 52-54, 56-64) - partial

Idem as subject 1, but restricted to a gene comprising SEQ ID NO:8.

6. Claims: (65) - complete

Use of interleukin 12 as an adjuvant in a C. difficile vaccine.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

7. Claims: (66) - partial

Use of humanised antibodies for passive vaccination of an individual with C. difficile infection.

8. Claims: (66) - partial

Use of serum for passive vaccination of an individual with C. difficile infection.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Please notice that:

1. The translations of the ORFs contained in appendices 1-8 are not included in the sequence listing and, therefore, the one corresponding to the first invention has not been searched. In case the applicant decided to pay additional fees, he should be aware of the fact that the same will apply to the other inventions..

2. Claims 39 and 56 refer to SEQ ID NOs:3-10 as 'amino acid sequences' but, actually, they are nucleotidic sequences.

3. The sequence numbering is confusing. The sequence identity numbers mentioned in the description and claims do not correspond with those of the sequence listing (example: SEQ ID NO:1 of the description is a peptide which appears under SEQ ID NO:9 of the sequence listing).

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/IE 02/00017

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9920384 A	29-04-1999	AU 1108299 A	10-05-1999
		CA 2307331 A1	29-04-1999
		EP 1024826 A1	09-08-2000
		WO 9920384 A1	29-04-1999
		US 6214341 B1	10-04-2001
		US 2001051153 A1	13-12-2001

Form PCT/ISA/210 (patent family annex) (July 1992)

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PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:

see form PCT/ISA/220

PCT

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY
(PCT Rule 43*bis*.1)

Date of mailing
(day/month/year) see form PCT/ISA/210 (second sheet)

Applicant's or agent's file reference
see form PCT/ISA/220

FOR FURTHER ACTION
See paragraph 2 below

International application No.	International filing date (day/month/year)	Priority date (day/month/year)
PCT/GB2005/002607	01.07.2005	02.07.2004

International Patent Classification (IPC) or both national classification and IPC
C07K16/12, A61K39/395

Applicant
NEUTEC PHARMA PLC

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☒ Box No. II Priority
- ☒ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☐ Box No. VIII Certain observations on the international application

2. FURTHER ACTION

If a demand for international preliminary examination is made, this opinion will usually be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA"). However, this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of three months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

3. For further details, see notes to Form PCT/ISA/220.

Name and mailing address of the ISA:

European Patent Office
D-80298 Munich
Tel. +49 89 2399 - 0 Tx: 523656 epmu d
Fax: +49 89 2399 - 4465

Authorized Officer:

Giebeler, K

Telephone No. +49 89 2399-8546

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/GB2005/002607

Box No. I Basis of the opinion

1. With regard to the language, this opinion has been established on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   ☐ This opinion has been established on the basis of a translation from the original language into the following language , which is the language of a translation furnished for the purposes of international search (under Rules 12.3 and 23.1(b)).

2. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, this opinion has been established on the basis of:

   a. type of material:

      ☒ a sequence listing

      ☐ table(s) related to the sequence listing

   b. format of material:

      ☒ in written format

      ☒ in computer readable form

   c. time of filing/furnishing:

      ☒ contained in the international application as filed.

      ☒ filed together with the international application in computer readable form.

      ☐ furnished subsequently to this Authority for the purposes of search.

3. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

4. Additional comments:

Box No. II Priority

1. ☒ The validity of the priority claim has not been considered because the International Searching Authority does not have in its possession a copy of the earlier application whose priority has been claimed or, where required, a translation of that earlier application. This opinion has nevertheless been established on the assumption that the relevant date (Rules 43*bis*.1 and 64.1) is the claimed priority date.

2. ☐ This opinion has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid (Rules 43*bis*.1 and 64.1). Thus for the purposes of this opinion, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/GB2005/002607

Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application,

☒ claims Nos. 1,2,10,11,18-32

because:

☒ the said international application, or the said claims Nos. 29-32 relate to the following subject matter which does not require an international preliminary examination *(specify)*:

see separate sheet

☐ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. are so unclear that no meaningful opinion could be formed *(specify)*:

☒ the claims, or said claims Nos. 18-28 (all partially) are so inadequately supported by the description that no meaningful opinion could be formed.

☒ no international search report has been established for the whole application or for said claims Nos. 1,2,10,11,18-20,26-32 (all partially)

☐ the nucleotide and/or amino acid sequence listing does not comply with the standard provided for in Annex C of the Administrative Instructions in that:

the written form	☐	has not been furnished
	☐	does not comply with the standard
the computer readable form	☐	has not been furnished
	☐	does not comply with the standard

☐ the tables related to the nucleotide and/or amino acid sequence listing, if in computer readable form only, do not comply with the technical requirements provided for in Annex C-bis of the Administrative Instructions.

☐ See separate sheet for further details

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/GB2005/002807

Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-11,13-32
	No:	Claims	12
Inventive step (IS)	Yes:	Claims	1-11,18-32
	No:	Claims	13-17
Industrial applicability (IA)	Yes:	Claims	1-26
	No:	Claims	

2. Citations and explanations

see separate sheet

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
Rad: 07-003888- -00000-0000
Fec: 2007-01-16 17:28:52 Dep: 2030 NUEVAS CREACIONES
Tra: 11 PATENTE PCT
Eve: 370 FASE NACIONAL
Act: 411 PRESENTACION Folios: 156

Industria y Comercio
SUPERINTENDENCIA

REQUISITOS MINIMOS PARA ADMISION A TRAMITE
PCT

PATENTE DE INVENCION [✓] MODELO DE UTILIDAD [ ]

- Indicación de que se solicita la concesión de una patente [✓]
- Datos de identificación del solicitante o de la persona que presenta la solicitud [✓]
- Descripción de la invención [✓]
- Dibujos de ser estos pertinentes [ ]
- Comprobante de pago de las tasas establecidas [✓]

COMPLETA [✓] INCOMPLETA [ ]

DISEÑO INDUSTRIAL [ ]

- Indicación de que se solicita el registro de Diseño Industrial [ ]
- Datos de identificación del solicitante o de la persona que presenta la solicitud [ ]
- Representación gráfica y fotográfica del Diseño Industrial o muestra del material que incorpora el diseño [ ]
- Comprobante de pago de las tasas establecidas [ ]

COMPLETA [ ] INCOMPLETA [ ]

ESQUEMA DE TRAZADO [ ]

- Indicación de que se solicita el registro de un Esquema de Trazado [ ]
- Datos de identificación del solicitante o de la persona que presenta la solicitud [ ]
- Representación gráfica del esquema trazado [ ]
- Comprobante de pago de las tasas establecidas [ ]

COMPLETA [ ] INCOMPLETA [ ]

____
Firma Responsable

Fecha____________________

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Bogotá, D.C., Colombia

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REPUBLICA DE COLOMBIA

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

Bogotá,
2020

Doctor(a)
RODRIGUEZ DILIA MARIA

REFERENCIA	Radicación No.	07 3868
	Solicitud internacional	PCT/GB2005/002607
	Fecha inicio fase nacional	02/02/2007
	Trámite	11
	Evento	379
	Actuación	430
	Oficio No.	1967

Como resultado del examen de forma, realizado con fundamento en el artículo 38 de la Decisión 486 de la Comisión de la Comunidad Andina, artículo 27 del Tratado de Cooperación en Materia de Patentes (PCT), regla 51 bis del Reglamento y Circular Única de la Superintendencia de Industria y Comercio, se le comunica que:

1. La solicitud no contiene la copia del documento en que consta la cesión del derecho a la patente del inventor al solicitante o a su causante. (Art. 26-k) Decisión 486.

2. La solicitud no contiene el poder debidamente otorgado, con el lleno de los requisitos exigidos por los artículos 63 y subsiguientes del Código de Procedimiento Civil. Adicionalmente, deberá presentar el documento que acredita la existencia y representación legal de la sociedad solicitante, cuando ésta fuere persona jurídica.

En cumplimiento de lo dispuesto por el artículo 39 de la Decisión 486, se le requiere para que dentro del término de dos meses siguientes a la fecha de notificación del presente oficio, complete los requisitos anteriormente enunciados. En caso de no dar cumplimiento al presente requerimiento, la solicitud se considerará abandonada y perderá su prelación.

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

ALIX CARMENZA CESPEDES DE VERGEL
Jefe de la división de Nuevas Creaciones

El anterior requerimiento fue notificado por fijación en lista de fecha 09 FEB. 2007
adpa10