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(54) **Title:** SHIGELLA IPAD PROTEIN AND ITS USE AS A VACCINE AGAINST SHIGELLA INFECTION

(57) **Abstract:** The present invention relates to compositions and methods for blocking entry of Shigella into a cell of an animal, to therefore providing protection against, or reduce the severity of Shigella infections. More particularly it relates to the use of the IpaD protein obtained from natural sources and/or through synthesis or recombinant technology, and conjugates thereof to induce neutralizing antibodies having protective activity against several serotypes of Shigella, in particular S. flexneri. The composition of the invention is useful to prevent and/or treat shigellosis caused by a bacterium of the Shigella family.

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SHIGELLA IPAD PROTEIN AND ITS USE AS A VACCINE AGAINST SHIGELLA INFECTION

FIELD OF THE INVENTION

The present invention relates to compositions and methods for blocking entry of Shigella into a cell of an animal, to therefore providing protection against, or reduce the severity of Shigella infections. More particularly it relates to the use of the IpaD protein obtained from natural sources and/or through synthesis or recombinant technology, and conjugates thereof to induce neutralizing antibodies having protective activity against several serotypes of *Shigella*, in particular *S. flexneri*. The composition of the invention is useful to prevent and/or treat shigellosis caused by a bacterium of the Shigella family.

BACKGROUND OF THE INVENTION

Many Gram-negative pathogenic bacteria use a type three secretion (T3S) system to interact with cells of their host. Each T3S system consists of a secretion apparatus (T3SA) that spans the bacterial envelope and extends on the bacterial surface, translocators that transit through the T3SA and insert into the membrane of the host cell where they form a pore, effectors that transit through the T3SA and the translocator pore to reach the cell cytoplasm, specific chaperones that associate with translocators and effectors in the bacterial cytoplasm and transcriptional regulators. Approximately 15 proteins are required for assembly of the T3SA.

Bacteria belonging to the *Shigella* family are the causative agents of bacillary dysentery in humans [1]. Genes required for entry of bacteria into epithelial cells and inducing apoptosis in macrophages are clustered in a 30-kb region, designated the entry region, of a 220-kb virulence plasmid. The entry region contains *mxi* and *spa* genes encoding components of the T3SA, the *ipaA*, *B*, *C* and *D*, *ipgB1*, *ipgD* and

icsB genes encoding proteins that transit through the T3SA, the *ipgA*, *ipgC*, *ipgE* and *spa15* genes encoding chaperones, and the *virB* and *mxiE* genes encoding transcriptional regulators [2].

The T3SA, which is weakly active in bacteria growing in broth, is activated upon contact of bacteria with epithelial cells [3]. Inactivation of *ipaB*, *ipaC* or *ipaD*, as well as most *mxi* and *spa* genes, abolishes the ability of bacteria to enter epithelial cells, induce apoptosis in macrophages and express contact hemolytic activity. IpaB and IpaC contain hydrophobic segments and remained associated with the membrane of lyzed erythrocytes, suggesting that these two proteins are components of the *S. flexneri* translocator. In addition, effector functions have been proposed for IpaB and IpaC [4,5,6,7,8]. Inactivation of *ipaB* and *ipaD*, but not *ipaC*, leads to a deregulated, i. e. constitutively active, T3SA, suggesting that IpaB and IpaD play a role in maintaining the T3SA inactive in the absence of inducers [9, 10]. A small proportion of IpaD is associated with the bacterial envelope [9, 11]. Picking and collaborators [12] reported that the role of IpaD in the control of the T3SA activity can be separated from its role in entry of bacteria into epithelial cells.

To get further insights on the structure of the needle complex, the inventors performed an immuno-electron microscopic analysis on bacteria treated with the cross-linking agent BS^3, both on entire bacteria and on the mildly purified needle complex (NC). The inventors present evidence that IpaD is a component of the NC localized at the tip of the needle and that antibodies raised against IpaD have an inhibitory effect on entry of *S. flexneri* into epithelial cells.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1: Electron micrographs of negatively stained bacteria treated with BS^3. Arrows indicate distinct densities at the tip of needles protruding from the bacterial surface. The bar represents 100 nm.

Fig. 2: Projection maps (class-sums) of the needle part of NCs obtained by single

particle analysis. (A-B) maps of NCs isolated from wild-type, treated with BS^3 (A-C); from wild-type without BS^3 treatment (D-F) and from an *ipaD* mutant strain with BS^3 treatment (G-I). The arrows point to remnant densities attached to needle parts prepared without BS^3 -treatment. The bar represents 10 nm.

Fig. 3: Immunobloting analysis of purified NCs. Whole cell extracts (WCE) and cross linked (+CR) and non-cross linked (-CR) NCs purified from wild-type and IpaD deficient strains were analyzed by SDS-PAGE and immunoblotting using antibodies specific to MxiJ, MxiN, and IpaD. IpaD was only enriched to WCE level in NCs prepared from cross-linked wild-type bacteria. MxiJ is a positive control to demonstrate intact T3SA. MxiN, a cytoplasmic component of T3S, is a positive control to demonstrate contamination by non-bound cytoplasmic proteins.

Fig. 4: IpaD localization by immuno-electron microscopy. NCs purified from BS^3-treated wild-type bacteria were incubated with anti-IpaD antibodies and negatively stained (A-D). The average image of 250 NCs purified from bacteria not treated with BS^3 is shown in E for comparison. The bar represents 10 nm.

Fig. 5: Invasion assay of epithelial cells by wild-type *S. flexneri*. Bacteria were incubated with serial dilutions of the anti-IpaD or anti-IpaB polyclonal antibodies and intracellular, gentamycin-resistant bacteria were counted by plating cell lysates. The efficiency of entry in each condition is expressed with respect to that of the wild-type strain treated with PBS. The values are the means of at least three independent experiments, and the error bars indicate standard deviations.

Fig. 6: Prior art amino acid sequence of the IpaD protein of a Shigella strain (GenBank accession number AL391753).

Fig. 7: Prior art nucleic acid sequence encoding the IpaD protein of Fig. 6 (GenBank accession number AL391753).

DESCRIPTION OF THE INVENTION

The inventors have surprisingly found that IpaD-specific neutralizing antibodies can block the entry of Shigella into permissive cells, such as epithelial cells and that this neutralizing effect is observed on different Shigella serotypes. In this connection, the present invention specifically relates to the use of the IpaD protein, the polynucleotide encoding same or anti-IpaD neutralizing antibodies in the preparation of compositions and elaboration of methods to elicit a cross protection against Shigella infection.

Definitions

The terms "animal" or "host" refer to any animal susceptible or known to be infected by a *Shigella* strain, such as *S. flexneri*. Specifically, the animal consists of a human.

The term "permissive cell" refers to a cell that can be infected by a Shigella strain. For instance, such a cell may be, but not limited to an epithelial cell or cells of the immune systems, particularly those that are targets for Shigella invasion in the intestinal mucosal immune system, such as dendritic cells and monocytes/macrophages, but also B and T lymphocytes that may undergo injection of Shigella effectors through the Type III secretion system, even if this does not lead to their invasion.

The term "treating" refers to a process by which the symptoms of an infection or a disease associated with a *Shigella* strain are alleviated or completely eliminated. As used herein, the term "preventing" refers to a process by which symptoms of an infection or a disease associated with a *Shigella* strain are obstructed or delayed.

The term "protective response" means prevention of onset of a *Shigella* - associated disease or an infection caused by a *species* or lessening the severity of such a disease existing in an animal.

The expression "an acceptable carrier" means a vehicle for containing the elements of the composition contemplated by the present invention that can be

administered to an animal host without adverse effects. Suitable carriers known in the art include, but are not limited to, gold particles, sterile water, saline, glucose, dextrose, or buffered solutions. Carriers may include auxiliary agents including, but not limited to, diluents, stabilizers (i.e., sugars and amino acids), preservatives, wetting agents, emulsifying agents, pH buffering agents, viscosity enhancing additives, colors and the like.

A "functional derivative", as is generally understood and used herein, refers to a protein/peptide sequence that possesses a functional biological activity that is substantially similar to the biological activity of the whole IpaD protein/peptide sequence. In other words, it preferably refers to a polypeptide or fragment(s) thereof that substantially retain(s) the capacity of eliciting the production of anti-IpaD neutralizing antibodies against a *S.* strain infection when said functional derivative is administered to an animal.

A "functional fragment", as is generally understood and used herein, refers to a nucleic acid sequence that encodes for a functional biological activity that is substantially similar to the biological activity of the whole IpaD nucleic acid sequence. In other words, and within the context of the present invention, it preferably refers to a nucleic acid or fragment(s) thereof that substantially retains the capacity of encoding a IpaD polypeptide/protein which elicits the production of anti-IpaD neutralizing antibodies against a *Shigella* strain infection when administered to an animal.

The term "Shigella serotype" refers to the four groups of Shigella that are identified by a capital letter from A-D: Shigella dysenteriae (A) Shigella flexneri (B), Shigella boydii (C), and Shigella sonnei (D). They respectively comprise: Group A: 8 serotypes, Group B: 11 serotypes and subserotypes, Group C: 11 serotypes, Group D: 1 serotype. For instance, a Shigella serotype may be, but not limited to, Shigella flexneri 2a, 1b and 3a, Shigella dysenteriae 1 and Shigella sonnei.

By the term "neutralizing" or "blocking", it is referred to the ability of the anti-IpaD antibodies of the invention to specifically bind to the IpaD protein and to interfere with the biological function of the IpaD protein therefore blocking, for

instance, the capacity of the bacterium to deliver its effectors of virulence to the target cells. In other words, such antibodies advantageously disarm the pathogen and make it both unable to cause lesions, and incapable to resist efficiently to host immune defences.

Compositions of the invention

In one aspect, the invention provides a composition for the treatment and/or the prevention of a Shigella infection. The invention also provides a composition for blocking entry of at least one Shigella serotype into a permissive cell. These contemplated compositions of the invention comprise at least one of the following elements:

- an IpaD polypeptide or functional derivative thereof;
- a polynucleotide encoding an IpaD polypeptide or a functional fragment thereof;
- an anti-IpaD neutralizing antibody.

As one skilled in the art may appreciate, the compositions of the invention advantageously provide a cross-protection against more than one Shigella serotype when administered to a host. In other words, the compositions of the invention prevent or substantially reduce the entry into a permissive cell of more than one Shigella serotype.

The IpaD polypeptide or functional derivative thereof contemplated by the present invention has for instance an amino acid sequence which is identical or substantially identical to the amino acid sequence having the GenBank accession number AL391753, and depicted herein as SEQ ID. NO:1 (see Fig. 6). In the case of a functional derivative of the IpaD polypeptide, one may use the plasmid pMal-IpaD deposited at the CNCM on October 10, 2007 under accession number I-3839. This plasmid encodes a fragment of the IpaD protein starting at codon 130 of the IpaD protein.

By "substantially identical" when referring to an amino acid sequence, it will be understood that the polypeptide contemplated by the present invention has, for instance, an amino acid sequence having at least 75% identity, or 85% identity or even 95% identity to part or all of the sequence shown in SEQ ID NO:1.

The polynucleotide or functional fragment thereof contemplated by the present invention codes for the IpaD polypeptide or a functional derivative thereof as defined above. For instance, such a polynucleotide has a nucleotide or nucleic acid sequence which is identical or substantially identical to the nucleotide sequence having GenBank accession number AL391753 and depicted herein as SEQ ID NO:2 (see Fig. 7). In the case of a functional fragment of the IpaD polynucleotide, one may use the plasmid pMal-IpaD as defined above.

By "substantially identical" when referring to a nucleotide or polynucleotide sequence, it will be understood that the polynucleotide contemplated by the present invention has, for instance, a nucleic acid sequence which is at least 65% identical, or 80% identical, or even 95% identical to part or all of the sequence shown in SEQ ID NO:2.

Techniques for determining nucleic acid and amino acid "sequence identity" also are known in the art. Typically, such techniques include determining the nucleotide sequence of the mRNA for a gene, the DNA sequence itself, and/or determining the amino acid sequence encoded thereby, and comparing these sequences to a second nucleotide or amino acid sequence. In general, "identity" refers to an exact nucleotide-to-nucleotide or amino acid-to-amino acid correspondence of two polynucleotides or polypeptide sequences, respectively. Two or more sequences (polynucleotide or amino acid) can be compared by determining their "percent identity." The percent identity of two sequences, whether nucleic acid or amino acid sequences, is the number of exact matches between two aligned sequences divided by the length of the shorter sequences and multiplied by 100. An approximate alignment for nucleic acid sequences is provided by the local homology algorithm of Smith and Waterman, Advances in Applied Mathematics 2:482-489 (1981). This algorithm can be applied to amino acid sequences by using the scoring

matrix developed by Dayhoff, Atlas of Protein Sequences and Structure, M. O. Dayhoff ed., 5 suppl. 3:353-358, National Biomedical Research Foundation, Washington, D.C., USA, and normalized by Gribskov, Nucl. Acids Res. 14(6):6745-6763 (1986). An exemplary implementation of this algorithm to determine percent identity of a sequence is provided by the Genetics Computer Group (Madison, Wis.) in the "BestFit" utility application. The default parameters for this method are described in the Wisconsin Sequence Analysis Package Program Manual, Version 8 (1995) (available from Genetics Computer Group, Madison, Wis.). A preferred method of establishing percent identity in the context of the present invention is to use the MPSRCH package of programs copyrighted by the University of Edinburgh, developed by John F. Collins and Shane S. Sturrok, and distributed by IntelliGenetics, Inc. (Mountain View, Calif.). From this suite of packages the Smith-Waterman algorithm can be employed where default parameters are used for the scoring table (for example, gap open penalty of 12, gap extension penalty of one, and a gap of six). From the data generated the "Match" value reflects "sequence identity." Other suitable programs for calculating the percent identity or similarity between sequences are generally known in the art, for example, another alignment program is BLAST, used with default parameters. For example, BLASTN and BLASTP can be used using the following default parameters: genetic code=standard; filter=none; strand=both; cutoff=60; expect=10; Matrix BLOSUM62; Descriptions=50 sequences; sort by=HIGH SCORE; Databases=non-redundant, GenBank+EMBL+DDBJ+PDB+GenBank CDS translations+Swiss protein+Spupdate+PIR.

The neutralizing antibodies contemplated by the invention which specifically bind to the IpaD protein may be prepared by a variety of methods known to one skilled in the art. For example, the IpaD polypeptide may be administered to an animal in order to induce the production of polyclonal antibodies. Alternatively, the anti-IpaD neutralizing antibodies used as described herein may be monoclonal antibodies, which are prepared using known hybridoma technologies (see, e.g., Hammerling *et al.*, In Monoclonal Antibodies and T-Cell Hybridomas, Elsevier, NY,

1981). With respect to contemplated antibodies of the present invention, the term "specifically binds to" refers to antibodies that bind with a relatively high affinity to one or more epitopes of the IpaD polypeptide, but which do not substantially recognize and bind molecules other than the IpaD polypeptide. As used herein, the term "relatively high affinity" means a binding affinity between the antibody and the IpaD polypeptide of at least 10^6 M^{-1}, and preferably of at least about 10^7 M^{-1} and even more preferably 10^8 M^{-1} to 10^{10} M^{-1}. Determination of such affinity is preferably conducted under standard competitive binding immunoassay conditions which are common knowledge to one skilled in the art.

It is understood that it is within one's knowledge in the field to screen and identify antibodies that neutralize the entry of Shigella into a cell.

In a preferred embodiment, the composition of the invention further comprises an adjuvant. As used herein, the term "adjuvant" means a substance added to the composition of the invention to increase the composition's immunogenicity. The mechanism of how an adjuvant operates is not entirely known. Some adjuvants are believed to enhance the immune response (humoral and/or cellular response) by slowly releasing the antigen, while other adjuvants are strongly immunogenic in their own right and are believed to function synergistically. Known adjuvants include, but are not limited to, oil and water emulsions (for example, complete Freund's adjuvant and incomplete Freund's adjuvant), Corytzebactei-ium parvuin, Quil A, cytokines such as IL12, Emulsigen-Plus®, Bacillus Calmette Guerin, aluminum hydroxide, glucan, dextran sulfate, iron oxide, sodium alginate, Bacto Adjuvant, certain synthetic polymers such as poly amino acids and co-polymers of amino acids, saponin, paraffin oil, and muramyl dipeptide. Adjuvants also encompass genetic adjuvants such as immunomodulatory molecules encoded in a co-inoculated DNA, or as CpG oligonucleotides. The coinoculated DNA can be in the same plasmid construct as the plasmid immunogen or in a separate DNA vector.

According a preferred embodiment of the invention, the compositions may further comprise a polyosidic antigen, such as those described in WO 2005/003995. For instance, contemplated polyosidic antigens may be, but not limited to synthetic

polysaccharides corresponding to the O-side chains (constitutives of the serotypes) of strains of interest, particularly those serotypes whose high prevalence is preferably considered: Shigella flexneri 2a, 1b and 3a, Shigella dysenteriae 1 and Shigella sonnei. The contemplated polyosidic antigens may also be detoxified lipopolysaccharide (LPS) extracted from bacterial cultures of similar serotypes.

Methods of treatment and compositions

The IpaD polypeptide or functional derivatives, the polynucleotide or functional fragments encoding same and antibodies contemplated by the invention may be used in many ways in the treatment and/or prevention of *Shigella* infection, or in the blocking entry of Shigella strains into a permissive cell.

For instance, and according to an aspect of the invention, the IpaD polypeptide may be used as immunogens for the production of specific anti-IpaD neutralizing antibodies. As previously mentioned, suitable anti-IpaD neutralizing antibodies may be determined using appropriate known screening methods, for example by measuring the ability of a particular antibody to neutralize or block a *Shigella* strain to enter into a cell.

According to another aspect, the polynucleotides encoding an IpaD polypeptide or derivatives thereof may be used in a DNA immunization method so as to produce anti-IpaD neutralizing antibodies. That is, they can be incorporated into a vector which is replicable and expressible upon injection thereby producing the antigenic polypeptide *in vivo*. For example polynucleotides may be incorporated into a plasmid vector under the control of the CMV promoter which is functional in eukaryotic cells. Preferably the vector is injected intramuscularly.

The use of a polynucleotide of the invention in genetic immunization will preferably employ a suitable delivery method or system such as direct injection of plasmid DNA into muscles [Wolf et al. H M G (1992) 1: 363, Turnes et al., Vaccine (1999), 17 : 2089, Le et al. , Vaccine (2000) 18 : 1893, Alves et al. , Vaccine (2001)19 : 788], injection of plasmid DNA with or without adjuvants [Ulmer et al. , Vaccine (1999) 18: 18, MacLaughlin et al. , J. Control Release (1998) 56: 259,

Hartikka et al. , Gene Ther. (2000) 7: 1171-82, Benvenisty and Reshef, PNAS USA (1986) 83: 9551, Singh et al. , PNAS USA (2000) 97: 811], targeting cells by delivery of DNA complexed with specific carriers [Wa et al. , J Biol Chem (1989) 264: 16985, Chaplin et al., Infect. Immun. (1999) 67:6434], injection of plasmid complexed or encapsulated in various forms of liposomes [Ishii et al. , AIDS Research and Human Retroviruses (1997) 13: 142, Perrie et al., Vaccine (2001) 19:3301], administration of DNA with different methods of bombardment [Tang et al., Nature (1992) 356: 152, Eisenbraun et al. , DNA Cell Biol (1993) 12: 791, Chen et al. , Vaccine (2001) 19:2908], and administration of DNA with lived vectors[Tubulekas et al. , Gene (1997) 190: 191, Pushko et al., Virology (1997) 239: 389, Spreng et al. FEMS (2000) 27: 299, Dietrich et al., Vaccine (2001) 19: 2506].

A further aspect of the invention is the use of the specific anti-IpaD neutralizing antibodies for passive immunization.

Yet, another aspect of the present invention is to provide a method for treating and/or preventing a *Shigella* infection in an animal. The method of the invention comprises the step of administering to the animal a composition according to the invention.

Yet, a further aspect of the invention is to provide a method for blocking entry of at least one Shigella serotype into a permissive cell, comprising the step of allowing the formation of an immune complex by contacting an anti-IpaD neutralizing antibody with a Shigella strain capable of infecting said permissive cell, said immune complex preventing or substantially reducing entry of said Shigella strain in the permissive cell.

The amount of the components or the elements of the compositions of the invention is preferably a therapeutically effective amount. A therapeutically effective amount of the contemplated component is the amount necessary to allow the same to perform their immunological role (i.e. production of anti-IpaD neutralizing antibodies) without causing overly negative effects in the host to which the composition is administered. The exact amount of the components to be used and the composition to be administered will vary according to factors such as the type of

condition being treated, the type and age of the animal to be treated, the mode of administration, as well as the other ingredients in the composition.

The compositions of the invention may be given to an animal through various routes of administration. For instance, the compositions may be administered in the form of sterile injectable preparations, such as sterile injectable aqueous or oleaginous suspensions. These suspensions may be formulated according to techniques known in the art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparations may also be sterile injectable solutions or suspensions in non-toxic parenterally-acceptable diluents or solvents. They may be given parenterally, for example intravenously, intramuscularly or subcutaneously by injection, by infusion or *per os*. Suitable dosages will vary, depending upon factors such as the amount of each of the components in the compositions, the desired effect (short or long term), the route of administration, the age and the weight of the animal to be treated. Any other methods well known in the art may be used for administering the composition of the invention.

A further aspect of the invention is to provide kits for use within any of the above methods contemplated by the present invention. A kit may comprise two or more components necessary for performing a defined assay. Components may be compounds, reagents, containers and/or equipment. For example, one container within a kit may contain a monoclonal antibody or fragment thereof or polyclonal antibodies that specifically neutralize an IpaD protein. One or more additional containers may enclose elements, such as reagents or buffers, to be used in the assay. Other kits contemplated by the present invention may comprise at least one IpaD polypeptide or a polynucleotide encoding same, as described above, to direct a neutralizing immune response against the IpaD protein of Shigella.

The present invention will be more readily understood by referring to the following examples. These examples are illustrative of the wide range of applicability of the present invention and are not intended to limit its scope. Modifications and variations can be made therein without departing from the spirit and scope of the invention. Although any methods and materials similar or equivalent to those

described herein can be used in the practice for testing of the present invention, preferred methods and materials are described hereinafter.

EXAMPLES

Type III secretion (T3S) systems are used by numerous Gram-negative pathogenic bacteria to inject virulence proteins into animal and plant host cells. The core of the T3S apparatus, known as the needle complex, is composed of a basal body transversing both bacterial membranes and a needle protruding above the bacterial surface. In *Shigella flexneri*, IpaD is required to inhibit the activity of the T3S apparatus prior to contact of bacteria with host and has been proposed to assist translocation of bacterial proteins into host cells. The inventors investigated the localization of IpaD by electron microscopy analysis of cross-linked bacteria and mildly purified needle complexes. This analysis revealed the presence of a distinct density at the needle tip. A combination of single particle analysis, immuno-labeling and biochemical analysis, demonstrated that IpaD forms part of the structure at the needle tip. Anti-IpaD antibodies were shown to block entry of bacteria into epithelial cells.

General materials and methods

Bacterial strains and growth media

Strains used in this study are the wild-type *S. flexneri* 5 strain M90T-Sm [13], its *ipaD* derivative SF622 [14]. Bacteria were grown in tryptic casein soy broth (TSB) (Sigma) at 37°C.

Purification of needle complex (NC)

NCs were purified as described [15]. Bacteria in the exponential phase of growth in 1 l of TSB at 37°C were collected by centrifugation, resuspended in 25 ml of phosphate-buffered saline and incubated in the presence of 1 mM Bis (Sulfosuccinimidyl)suberate (BS^3) for 30 min at 37°C. The mixture was supplemented

with 100 mM Tris-HCl and incubated for 15 min at 37°C. BS^3-treated cultures were harvested and resuspended in an ice-cold lysis buffer (0.5 M sucrose, 20 mM Tris-HCl [pH 7.5], 2 mM EDTA, 0.5 mg/ml lysozyme) supplemented with 1 mM phenylmethylsulfonyl fluoride and incubated for 45 min at 4°C and for 15 min at 37°C. Resulting spheroplasts were incubated with 0.01% Triton X-100 for 30 min and treated with 4 mM $MgCl_2$ and 80 µg/ml DNAse (Sigma) for 20 min at 30°C. Debris were removed by centrifugation (20,000 *g* for 20 min at 4°C) and the membrane fraction was pelleted by centrifugation (110,000 *g* for 30 min at 4°C) and resuspended in TET buffer (20 mM Tris–HCl pH 7.5, 1 mM EDTA, 0.01% Triton X-100). Immunoblotting analysis was performed with antibodies raised against MxiJ, MxiN and IpaD as described [16].

Electron microscopy and image analysis

Whole cells and samples of purified NCs were negatively stained with 2% uranyl acetate on glow discharged carbon-coated copper grids. Electron microscopy was performed on a Philips CM120FEG equipped with a field emission gun operated at 120 kV. Images were recorded with a 4000 SP 4K slow-scan CCD camera at 80,000 x magnification at a pixel size (after binning the images) of 3.75 Å at the specimen level, with "GRACE" software for semi-automated specimen selection and data acquisition [17]. Single particle analysis including multi-reference and non-reference procedures, multivariate statistical analysis and classification was performed as described [15]. For immuno-labeling, purified NCs were incubated with affinity purified IpaD polyclonal antibodies (pAbs) at a final concentration of 0.132 ng/µl) for 1 hr at 20°C. Samples were stained with 2% uranyl acetate and observed as above.

Invasion assay

Two ml of cultures of wild-type or *mxiD* strains in the exponential phase of growth (OD_{600nm} of 0.4) were incubated in the presence of anti-IpaD (dilution 1/2000 to 1/50) or anti-IpaB (1/50) antibodies for 1 h at 37°C and bacteria were centrifuged

on plates containing 2 10^5 Hela cells for 10 min at 2000 g. After 1 h incubation at 37°C, cells were washed three times with 2 ml EBSS and incubated during 1 h with 2 ml MEM milieu containing 50 µg/ml gentamycin. After three washes with 2 ml EBSS, plates were incubated with a solution of deoxycholate 0.5% for 15 min at 20°C and cell lysates were diluted and plated on agar plates for colony counting.

Example 1: A distinctive structure at the tip of the T3SA needle

Protein purification procedures tend to select for most stable complexes that might not contain weakly associated subunits. The inventors recently showed that a Triton-X100 detergent concentration as low as 0.01% was sufficient to induce the release of NCs from the membrane (Sani *et al.*, 2006). To detect potentially labile subunits attached to the needle, the inventors performed a cross-linking step with BS^3 on bacteria prior to any purification. Electron microscopy analysis indicated that, following BS^3 treatment, most bacteria exhibited needle appendages with an additional density at the extremity of the tip (Fig. 1).

NCs were purified from BS^3-treated bacteria after detergent solubilization of membranes as described [18]. Preparations contained a sufficient number of NCs with the additional densities at the needle tip to perform a structural analysis. To calculate two-dimensional projection maps of isolated NCs, electron microscopy images were analysed by single particle analysis. The inventors selected several hundred images of NCs with a relatively straight and short needle appendage and a length close to 45 nm. The averaged NCs clearly showed the presence of a density around the needle tip, as well as the upper part of the basal body (Fig. 2A and 2B; see also Fig. 4E for a total view of a NC). However, NCs appeared a bit blurred after averaging as a result of variations in the needle length. Sharper features at the tip of the needle portion were obtained when projections were aligned and classified after masking the basal part (Fig. 2C). Striking features of the average map of cross-linked particles are the presence of densities at either side of the needle tip. In contrast, average maps of particles prepared from the wild-type strain without cross-linking showed needles lacking most of these densities (Fig. 2D-F). Faint densities are

visible in these samples at the same position where the strong densities were present in cross-linked preparations (arrows, Fig. 2E and 2F). These results suggest that, in the absence of cross-linking, most purified NCs lost the additional molecule(s) forming the density observed after cross-linking. To identify molecule(s) forming the density at the tip of the T3SA, the inventors performed similar experiments with an *ipaD* mutant lacking the IpaD expression. IpaD is required, together with IpaB, to maintain the T3SA inactive in the absence of inducers and a small proportion of IpaD is membrane associated [9]. NCs purified from the BS^3-treated *ipaD* mutant did not exhibit densities at either side of the needle tip (Fig. 2G-I), suggesting that IpaD is part of or required for assembly of this structural element.

Example 2: IpaD is present at the tip of the T3SA needle

To test whether IpaD constitutes the observed density, NCs purified from BS^3-treated wild-type bacteria were analyzed by SDS-PAGE and immunoblotting (Fig. 3). IpaD was enriched in NCs prepared from cross-linked wild-type bacteria, as compared to NCs prepared from non-cross-linked bacteria (Fig. 3, right panel, right lane), though small amounts also co-purifiy in non-cross linked preparation and thus corroborate the faint densities at the needle tip for averages of non cross linked NCs. MxiJ that is a major NC component is present in similar amounts in all preparations (Fig. 3). Control experiments using antibodies recognizing cytoplasmic components of the T3S system, such as MxiN, did not reveal any contamination of NCs by intracellular components (Fig. 3), indicating that the presence of IpaD in the preparations was not due to a contamination by cytoplasmic proteins.

To confirm that densities detected at the tip of NCs contained IpaD, the inventors performed immuno-staining using an anti-IpaD serum. Antibodies specifically bound to the tip of the needle in NCs prepared from BS^3 treated wild-type bacteria (Fig. 4 A-D). Some needles were also observed to be associated by their tip, presumably as a result of the interaction to divalent antibodies with two needles (lower left frame, Fig. 4D). In control experiments, no antibodies were found to bind the needle of NCs isolated from the *ipaD* mutant (data not shown).

Example 3: Anti-IpaD antibodies blocks entry of bacteria into epithelial cells

Since IpaD is required for entry of bacteria into epithelial cells [14] and since, as shown here, it is localized at the tip of the T3SA, the inventors investigated whether anti-IpaD antibodies might interfere with entry of bacteria into HeLa cells. Bacteria incubated with different concentrations of the anti-IpaD serum, or an anti-IpaB serum as a control, were used to infect HeLa cells. Exposure of bacteria to the anti-IpaD serum, but not to the anti-IpaB serum, inhibited bacterial entry in a dose-dependent manner (Fig. 5). Treatment with the anti-IpaD antibodies also inhibited entry of a *S. flexneri 2a* strain (data not shown).

General discussion regarding Example 1 to 3

The *S. flexneri* T3SA is activated upon contact of bacteria with epithelial cells and is deregulated by inactivation of *ipaB* or *ipaD*. It was proposed that these proteins are required to form a complex pluging the T3SA. Here, the inventors present evidence that IpaD is present at the tip of the needle. Transmission electron micrograph of surface exposed needles from cross-linked bacteria showed a distinctive structure present at the tip of the needle and immunoblot analysis of mildly purified NCs indicated that IpaD is copurified with the cross-linked NCs.

Calculated averages of NCs isolated from cross-linked wild-type bacteria showed distinct densities at either sides of the needle tip. This feature was not observed in NCs isolated from both the wild-type strain that had not been treated with the cross-linker and from the *ipaD* mutant treated with the cross-linker. Results of immunoelectron microscopy indicate that the observed density at the tip of the needle contains IpaD molecules. The exact configuration of the additional density, however, cannot be retrieved from 2D projections maps. The two additional masses have dimensions of about 7 x 7 nm. Since the size of IpaD is 37-kDa, several copies of IpaD are probably present in these structures. Indeed, IpaD has been proposed to form oligomers [19] IpaD presents some functional analogies with LcrV of *Yersinia enterocolitica*, inasmuch as the two proteins have similar sizes and are both required

for insertion of the proposed translocators, IpaB and IpaC in S. flexneri and YopB and UopD in *Y. enterocolitica*, in the membrane of host cells [9, 20, 21]. Recent data indicated that LcrV is localized at the tip of the T3SA needle [20]. The structure in *Yersinia* appears to be slightly different to that in *Shigella* with smaller protruding densities at the side and with a different tip.

The identification of a structural element containing IpaD at the tip of the T3SA needle provides further insights on the composition and structure of the *S. flexneri* T3SA. Very recently it was also demonstrated with biochemical methods that IpaD localizes to the T3SA needle tip, where it functions to control the secretion and proper insertion of translocators into host cell membranes [22]. The present single particle analysis, however, directly demonstrates the position of IpaD at the tip of the needle and adds credence to the hypothesis that IpaD acts as a plug to the T3SA prior to contact of bacteria with cells. As proposed for LcrV in *Yersinia*, IpaD might also facilitate insertion of components of the translocators within the cell membrane. The inhibition of entry of bacteria into HeLa cells by treatment with anti-IpaD neutralizing antibodies indicates that binding of antibodies to IpaD interferes with the function of the protein. LcrV has also been shown to be a protective antigen for plague disease in animal studies [23, 24]. Accordingly, IpaD represent an interesting target for the preparation of vaccines that would be effective against several serotypes of *Shigella*.

Example 4: **Protective effect of anti-IpaD antibodies *in vivo***

Intestinal iliac loops performed in the rabbit were inoculated with a suspension of 10^9 CFU of *Shigella flexneri* serotype 5a alone or were incubated in the presence of different dilutions of a rabbit polyclonal serum specific for IpaD. The anti-IpaD polyclonal serum was produced following an immunization with a functional derivative of the IpaD protein expressed by the pMal-IpaD expression vector deposited at the CNCM on October 10, 2007 under accession number I-3839. This model summarizes the set of lesions observed during shigellosis in humans following the rupture, the invasion and the inflammatory destruction of the intestinal epithelium by

this entero-invasive bacterium. These lesions are manifested through a combination of morphological alterations of the intestinal villi and edema, combined with an inflammatory cellular filtrate, in particular polynuclear neutrophils, and abscesses which eventually become ulcerated in the intestinal lumen. Overall, this gives rise to luminal mucopurulent exudates which are often invasive.

By comparing tissue destruction in the intestinal loops inoculated with bacteria alone with those having received bacteria in the presence of anti-IpaD polyclonal serum, a protective effect was observed which is dependent on the anti-IpaD antibody concentration. Indeed, when the serum used is non-diluted, no lesions are visible. However, when the serum used is in a 1/10 dilution, very discrete lesions appear, becoming clearer when a 1/100 dilution is used, all the while remaining less important than those observed with bacteria alone. No protection is observed with a control polyclonal serum directed to a non relevant protein. Therefore, the protection observed is specifically linked to the presence of anti-IpaD antibodies.

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WHAT IS CLAIMED IS:

1. A composition for blocking entry of at least one Shigella serotype into a permissive cell, comprising at least one of the following elements:

- an IpaD polypeptide or functional derivative thereof;
- a polynucleotide or functional fragment thereof encoding an IpaD polypeptide; and/or
- an anti-IpaD neutralizing antibody.

2. A composition for the treatment and/or the prevention of a Shigella infection, comprising at least one of the following elements:

- an IpaD polypeptide or functional derivative thereof;
- a polynucleotide or functional fragment thereof encoding an IpaD polypeptide; and/or
- an anti-IpaD neutralizing antibody.

3. The composition according to claim 1 or 2, wherein said IpaD polypeptide has an amino acid sequence identical or substantially similar to SEQID NO:1.

4. The composition according to claim 1 or 2, wherein said polynucleotide has a nucleotide sequence identical or substantially similar to SEQID NO:2.

5. The composition of any one of claims 1 to 4, wherein the anti-IpaD neutralizing antibody is polyclonal.

6. The composition of any one of claims 1 to 4, wherein the anti-IpaD neutralizing antibody is monoclonal.

7. The composition of any one of claims 1 to 6, further comprising a polyosidic antigen.

8. Use of at least one of the following element for blocking entry of at least one Shigella serotype into a permissive cell, comprising:

- an IpaD polypeptide or functional derivative thereof;
- a polynucleotide or functional fragment thereof encoding an IpaD polypeptide; and/or
- an anti-IpaD neutralizing antibody.

9. Use at least one of the following elements for the treatment and/or the prevention of a Shigella infection, comprising:

- an IpaD polypeptide or functional derivative thereof;
- a polynucleotide or functional fragment thereof encoding an IpaD polypeptide; and/or
- an anti-IpaD neutralizing antibody.

10. The use according to claim 8 or 9, wherein said IpaD polypeptide has an amino acid sequence identical or substantially similar to SEQ ID NO:1.

11. The use according to claim 8 or 9, wherein said polynucleotide has a nucleotide sequence identical or substantially similar to SEQ ID NO:2.

12. The use of any one of claims 8 to 11, wherein the anti-IpaD neutralizing antibody is polyclonal.

13. The use of any one of claims 8 to 11, wherein the anti-IpaD neutralizing antibody is monoclonal.

14. The use of any one of claims 8 to 13, further comprising a polyosidic antigen.

15. A kit for blocking entry of at least one Shigella serotype into a permissive cell, comprising:

- an IpaD polypeptide or functional derivative thereof;
- a polynucleotide or functional fragment thereof encoding an IpaD polypeptide; and/or
- an anti-IpaD neutralizing antibody.

16. A method for the treatment and/or the prevention of a Shigella infection in an animal, comprising the step of administering to said animal a composition as defined in any one of claims 1 to 7.

17. A method for blocking entry of at least one Shigella serotype into a permissive cell, comprising the step of allowing the formation of an immune complex by contacting an anti-IpaD neutralizing antibody with a Shigella strain capable of infecting said permissive cell, said immune complex preventing or substantially reducing entry of said Shigella strain in the permissive cell.

18. The method of claim 17, consisting of an *in vivo* method.

19. The method of claim 17 or 18, wherein the anti-IpaD neutralizing antibody is produced by a host comprising said permissive cell following administration to said host of an IpaD polypeptide or functional derivative thereof and/or of a polynucleotide or functional fragment thereof encoding an IpaD polypeptide.

20. The method according to claim 19, wherein said IpaD polypeptide has an amino acid sequence identical or substantially similar to SEQ ID NO:1.

21. The method according to claim 19, wherein said polynucleotide has a nucleotide sequence identical or substantially similar to SEQ ID NO:2.

22. The method of claim 17, wherein the anti-IpaD neutralizing antibody is provided to a host comprising said permissive cell by passively immunizing said host.

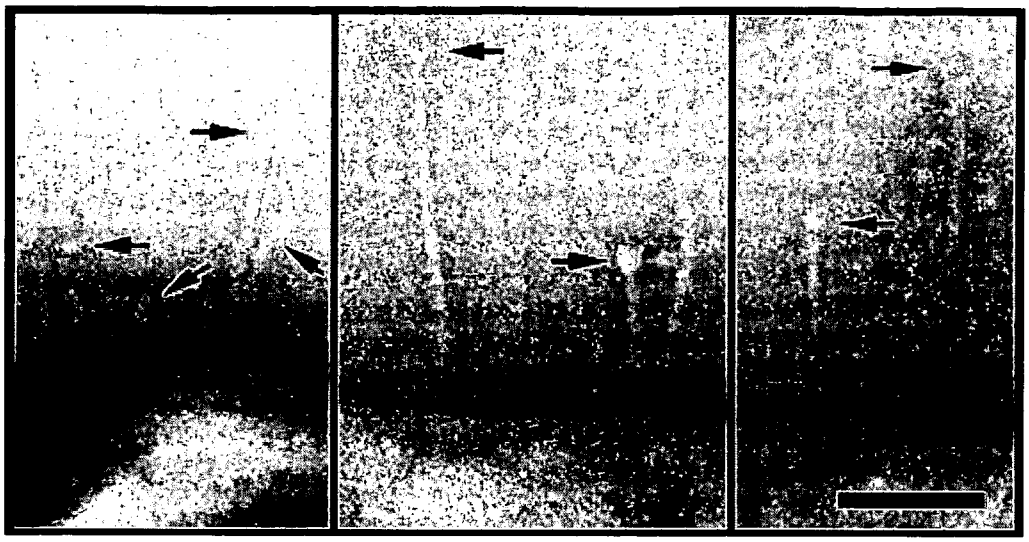

FIGURE 1

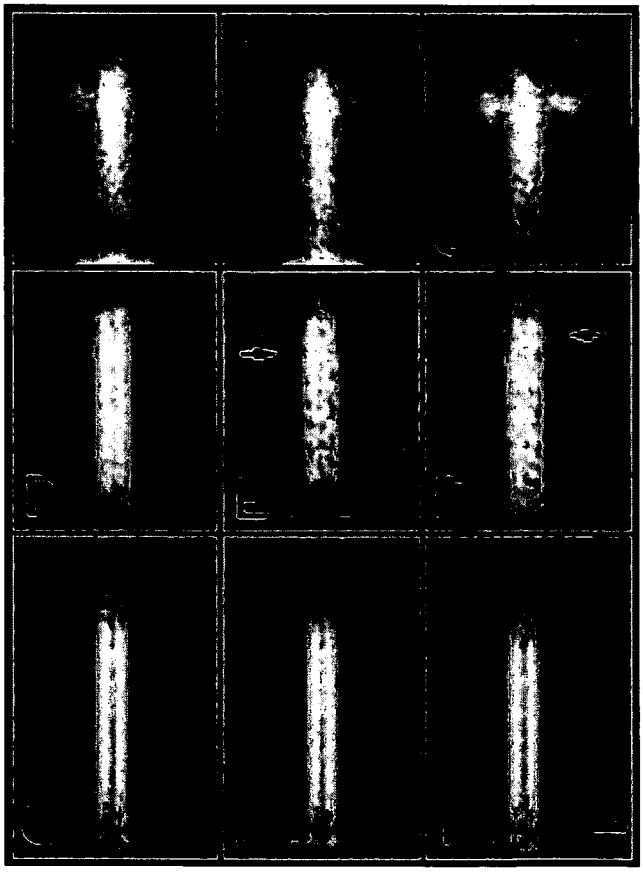

FIGURE 2

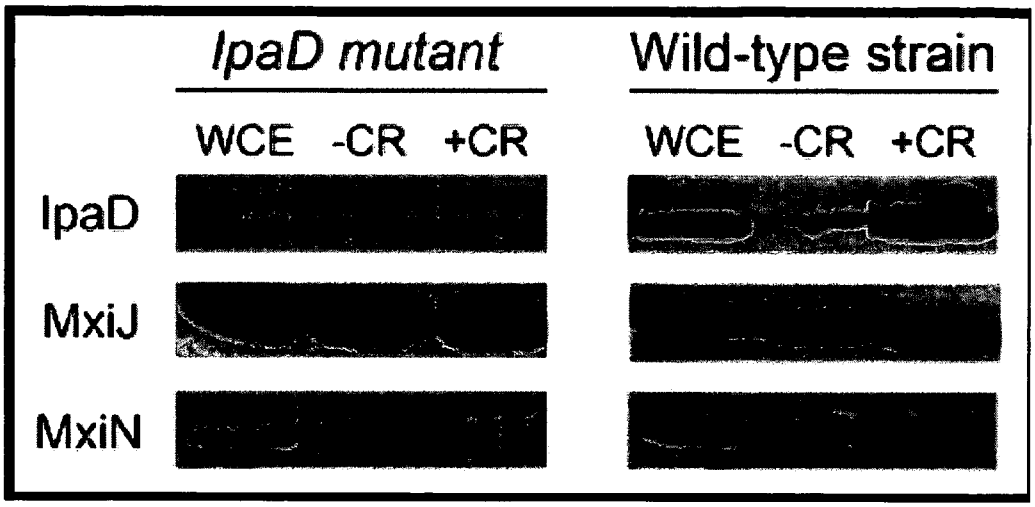
IpaD mutant
Wild-type strain
WCE -CR +CR
WCE -CR +CR
IpaD
MxiJ
MxiN

FIGURE 3

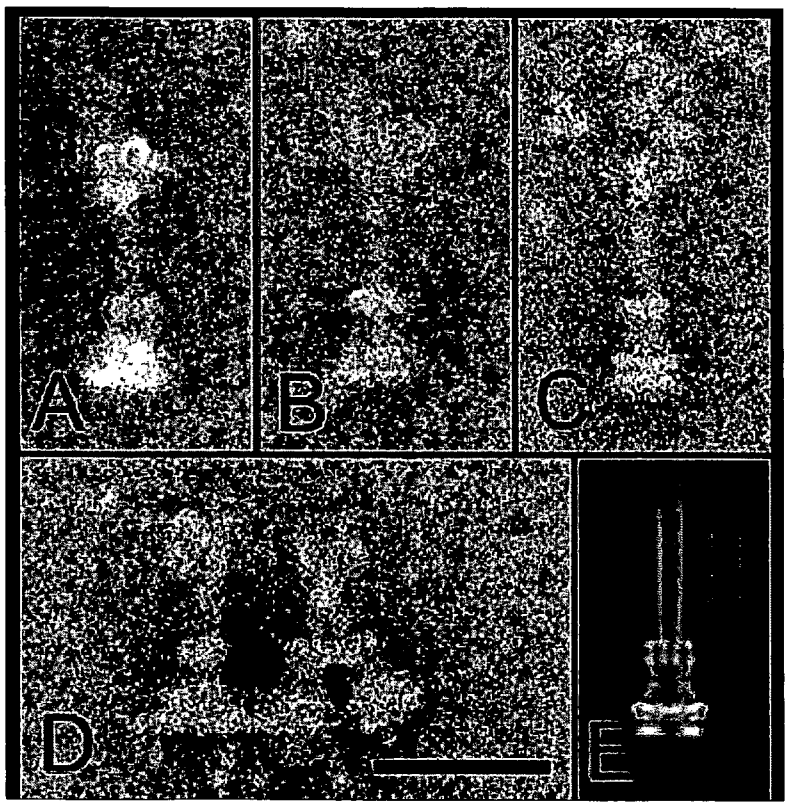
A
B
C
D
E

FIGURE 4

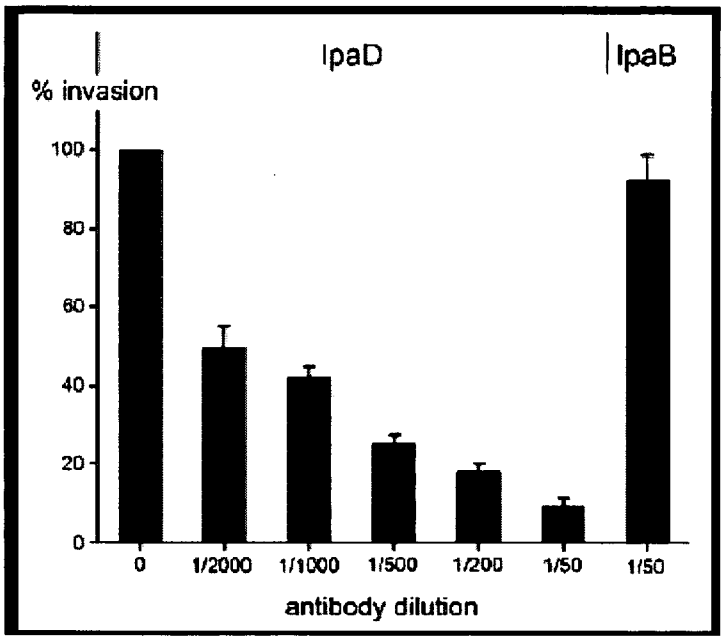

FIGURE 5

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MNITTLTNSI STSSFSPNNT NGSSTETVNS DIKTTTSSHP VSSLTMLNDT LHNIRTTNQA 60
LKKELSQKTL TKTSLEEIAL HSSQISMDVN KSAQLLDILS RNEYPINKDA RELLHSAPKE 120
AELDGDQMIS HRELWAKIAN SINDINEQYL KVYEHAVSSY TQMYQDFSAV LSSLAGWISP 180
GGNDGNSVKL QVNSLKKALE ELKEKYKDKP LYPANNTVSQ EQANKWLTEL GGTIGKVSQK 240
NGGYVVSINM TPIDNMLKSL DNLGGNGEVV LDNAKYQAWN AGFSAEDETM KNNLQTLVQK 300
YSNANSIFDN LVKVLSSTIS SCTDTDKLFL HF                               332
```

FIGURE 6

```
   1 tcagaaatgg agaaaaagtt tatctgtatc tgtacatgag cttattgtac tactcaaaac
  61 ctttactaaa ttatcaaaaa tactattggc attactgtat ttttgaacta aagtttgaag
 121 attatttttc attgtttcat cttcggcaga gaatccggca ttccatgcct gatattttgc
 181 attatctagc acaacctcgc catttccacc tagattatct aagcttttta acatattgtc
 241 tattggggtc atgtttatac tgacaacata tcccccgttt ttttgagata ccttgccgat
 301 tgttccacct aattctgtaa gccatttatt tgcttgttcc tgactaacag tattatttgc
 361 tggatatagc ggtttatctt tatatttttc cttgagttct tccaatgcct ttttaagcga
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 601 ttctctatga gatatcattt gatctccatc aagctcggct tctttcgggg ctgaatgtaa
 661 taattctctt gcgtctttat taattggata ttcgttcctg gaaagaatat ccaatagttg
 721 agcggattta tttacatcca tgctaatctg agatgaatgt aatgctattt cttctagcga
 781 tgttttagtc aacgtttttt gtgaaagctc tttctttaat gcctgatttg ttgttctgat
 841 attatgaagg gtgtcgttga gcatagtaag ggaacttaca ggatgagaac tggtcgttgt
 901 ttttatatca gaattaactg tttcggttga tgaaccgttg gtattgtttg gactgaatga
 961 tgaggtggaa atactattag tcagagttgt tatattcat
```

FIGURE 7

TRAITÉ DE BUDAPEST SUR LA RECONNAISSANCE INTERNATIONALE DU DÉPÔT DES MICRO-ORGANISMES AUX FINS DE LA PROCÉDURE EN MATIÈRE DE BREVETS

FORMULE INTERNATIONALE

DESTINATAIRE

INSTITUT PASTEUR
SERVICE DES BREVETS ET INVENTIONS
25-28, RUE DU DOCTEUR ROUX
75724 PARIS CEDEX 15

RÉCÉPISSÉ EN CAS DE DÉPÔT INITIAL
délivré en vertu de la règle 7.1 par
l'AUTORITÉ DE DÉPÔT INTERNATIONALE
identifiée au bas de cette page

NOM ET ADRESSE
DU DÉPOSANT

I. IDENTIFICATION DU MICRO-ORGANISME	
Référence d'identification donnée par le DÉPOSANT : **RM 161**	Numéro d'ordre attribué par l'AUTORITÉ DE DÉPÔT INTERNATIONALE : **CNCM I-3839**

II. DESCRIPTION SCIENTIFIQUE ET/OU DÉSIGNATION TAXONOMIQUE PROPOSÉE

Le micro-organisme identifié sous chiffre I était accompagné :

[X] d'une description scientifique

[X] d'une désignation taxonomique proposée

(Cocher ce qui convient).

III. RÉCEPTION ET ACCEPTATION

La présente autorité de dépôt internationale accepte le micro-organisme identifié sous chiffre I, qu'elle a reçu le **10 OCTOBRE 2007** (date du dépôt initial) [1].

IV. RÉCEPTION D'UNE REQUÊTE EN CONVERSION

La présente autorité de dépôt internationale a reçu le micro-organisme identifié sous chiffre I le (date du dépôt initial) [1] et a reçu une requête en conversion du dépôt initial en dépôt conforme au Traité de Budapest le (date de réception de la requête en conversion).

V. AUTORITÉ DE DÉPÔT INTERNATIONALE

Nom : **COLLECTION NATIONALE DE CULTURES DE MICROORGANISMES (CNCM)**	Signature(s) de la (des) personne(s) compétente(s) pour représenter l'autorité de dépôt internationale ou de l'(des) employé(s) autorisé(s) : **GEORGES WAGENER**
Adresse : **INSTITUT PASTEUR 25 RUE DU DOCTEUR ROUX 75724 PARIS CEDEX 15**	Date : **PARIS, LE 15 NOVEMBRE 2007**

[1] En cas d'application de la règle 6.4.d), cette date est la date à laquelle le statut d'autorité de dépôt internationale a été acquis.

Formule BP/4 (page unique)

TRAITÉ DE BUDAPEST SUR LA RECONNAISSANCE INTERNATIONALE DU DÉPÔT DES MICRO-ORGANISMES AUX FINS DE LA PROCÉDURE EN MATIÈRE DE BREVETS

FORMULE INTERNATIONALE

DESTINATAIRE

MADAME DANIELLE BERNEMAN
SERVICE DES BREVETS ET INVENTIONS
INSTITUT PASTEUR
25-28, RUE DU DOCTEUR ROUX
75724 PARIS CEDEX 15

NOM ET ADRESSE DE LA PARTIE À LAQUELLE LA DÉCLARATION SUR LA VIABILITÉ EST DÉLIVRÉE

DÉCLARATION SUR LA VIABILITÉ
délivrée en vertu de la règle 10.2 par
l'AUTORITÉ DE DÉPÔT INTERNATIONALE
identifiée à la page suivante

I. DÉPOSANT	II. IDENTIFICATION DU MICRO-ORGANISME
Nom : **INSTITUT PASTEUR** Adresse : **SERVICE DES BREVETS ET INVENTIONS** **25-28, RUE DU DOCTEUR ROUX** **75724 PARIS CEDEX 15**	Numéro d'ordre attribué par l'AUTORITÉ DE DÉPÔT INTERNATIONALE : **CNCM I-3839** Date du dépôt ou du transfert [1] : **10 OCTOBRE 2007**

III. DÉCLARATION SUR LA VIABILITÉ

La viabilité du micro-organisme identifié sous chiffre II a été contrôlée le **15 OCTOBRE 2007** [2]. À cette date, le micro-organisme

- [X] [3] était viable
- [ ] [3] n'était plus viable

[1] Indiquer la date du dépôt initial ou, si un nouveau dépôt ou un transfert ont été effectués, la plus récente des dates pertinentes (date du nouveau dépôt ou date du transfert).

[2] Dans les cas visés à la règle 10.2.a)ii) et iii), mentionner le contrôle de viabilité le plus récent.

[3] Cocher la case qui convient.

IV. CONDITIONS DANS LESQUELLES LE CONTRÔLE DE VIABILITÉ A ÉTÉ EFFECTUÉ [4]

V. AUTORITÉ DE DÉPÔT INTERNATIONALE

Nom : **COLLECTION NATIONALE DE CULTURES DE MICROORGANISMES (CNCM)**

Adresse : **INSTITUT PASTEUR**
25 RUE DU DOCTEUR ROUX
75724 PARIS CEDEX 15

Signature(s) de la(des) personne(s) compétente(s) pour représenter l'autorité de dépôt internationale ou de l'(des) employé(s) autorisé(s) : **GEORGES WAGENER**

Date : **PARIS, LE 15 NOVEMBRE 2007**

C. N. C. M. COLLECTION NATIONALE DE CULTURES DE MICROORGANISMES Institut Pasteur

[4] À remplir si cette information a été demandée et si les résultats du contrôle étaient négatifs.