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(54) GENES PNEUMOCOCCIQUES, PARTIES DE CES GENES, LEURS PRODUITS D'EXPRESSION, ET UTILISATIONS DE CES GENES, PARTIES ET PRODUITS

(54) PNEUMOCOCCAL GENES, PORTIONS THEREOF, EXPRESSION PRODUCTS THEREFROM, AND USES OF SUCH GENES, PORTIONS AND PRODUCTS

(57) On décrit des gènes pneumococciques, des parties de ces gènes, leurs produits d'expression, des utilisations de ces gènes, parties et produits, et notamment des gènes de Streptococcus pneumoniae, par exemple le gène codant la protéine de surface pneumococcique A (PspA), c'est-à-dire le gène pspA, le gène codant des protéines apparentées à la PspA, tels que des gènes apparentés à pspA, par exemple le gène codant la protéine de surface pneumococcique C, c'est-à-dire le gène pspC, des parties de ces gènes, des produits d'expression tirés de ceux-ci, ainsi que des utilisations de ces gènes, de leurs parties et produits d'expression.

(57) The present invention relates to pneumococcal genes, portions thereof, expression products therefrom and uses of such genes, portions and products; especially to genes of Streptococcus pneumoniae, e.g., the gene encoding pneumococcal surface protein A (PspA), i.e., the pspA gene, the gene encoding pneumococcal surface protein A-like proteins, such as pspA-like genes, e.g., the gene encoding pneumococcal surface protein C (PspC), i.e., the pspC gene, portions of such genes, expression products therefrom, and the uses of such genes, portions thereof and expression products therefrom.

CORRECTED VERSION*

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(54) Title: PNEUMOCOCCAL GENES, PORTIONS THEREOF, EXPRESSION PRODUCTS THEREFROM, AND USES OF SUCH GENES, PORTIONS AND PRODUCTS

(57) Abstract

The present invention relates to pneumococcal genes, portions thereof, expression products therefrom and uses of such genes, portions and products; especially to genes of *Streptococcus pneumoniae*, e.g., the gene encoding pneumococcal surface protein A (PspA), i.e., the *pspA* gene, the gene encoding pneumococcal surface protein A-like proteins, such as *pspA*-like genes, e.g., the gene encoding pneumococcal surface protein C (PspC), i.e., the *pspC* gene, portions of such genes, expression products therefrom, and the uses of such genes, portions thereof and expression products therefrom.

* (Referred to in PCT Gazette No. 31/1997, Section II)

PNEUMOCOCCAL GENES, PORTIONS THEREOF, EXPRESSION PRODUCTS THEREFROM, AND USES OF SUCH GENES, PORTIONS AND PRODUCTS

RELATED APPLICATIONS

This application is a continuation-in-part ("CIP"): of application Serial Nos. 08,529,055, filed September 15, 1995, 08/226,844, filed May 29, 1992, 08/093,907, filed May 29, 1992, 07/884,918, filed July 5, 1994 (corresponding to PCT/US93/05191); of application Serial No. 08/482,981, filed June 7, 1995; of application Serial No. 08/458,399, filed June 2, 1995; of application Serial No. 08/446,201, filed May 19, 1995 (as a CIP of USSN 08/246,636); of application Serial No. 08/246,636, filed May 20, 1994 (as a CIP of USSN 08/048,896, filed April 20, 1993 as a CIP of USSN 07/835,698, filed February 12, 1992 as a CIP of USSN 07/656,773); of application Serial 08/319,795, filed October 7, 1994 (as a CIP of USSN 08/246,636); of application Serial No. 08/072,070, filed June 3, 1993; of application Serial No. 07/656,773, filed February 15, 1991 (USSN 656,773 and 835,698 corresponding to Int'l application WO 92/1448); and, each of these applications, as well as each application, document or reference cited in these applications, is hereby incorporated herein by reference. Documents or references are also cited in the following text, either in a Reference List appended to certain Examples, or before the claims, or in the text itself; and, each of these documents or references is hereby expressly incorporated herein by reference.

FIELD OF THE INVENTION

This invention relates to pneumococcal genes, portions thereof, expression products therefrom and uses of such genes, portions and products; especially to genes of *Streptococcus pneumoniae*, e.g., the gene encoding pneumococcal surface protein A (PspA) (said gene being "*pspA*"), *pspA*-like genes, pneumococcal surface protein C (PspC) (said gene being "*pspC*"), portions of such genes, expression products therefrom, and the uses of such genes, portions thereof and expression products therefrom. Such uses include uses of the genes and portions thereof for obtaining expression products by recombinant techniques, as well as for detecting the presence of *Streptococcus pneumoniae* or strains thereof by detecting DNA thereof by hybridization or amplification (e.g., PCR) and hybridization techniques (e.g., obtaining DNA-containing sample, contacting same with genes or fragment under PCR, amplification and/or hybridization conditions, and detecting presence of or isolating hybrid or amplified product). The expression product uses include use in preparing antigenic, immunological or vaccine compositions, for eliciting antibodies, an immunological response (other than or additional to antibodies) or a protective response (including antibody or other immunological response by administering composition to a suitable host); or, the expression product can be for use in detecting the presence of *Streptococcus pneumoniae* by detecting antibodies to *Streptococcus pneumoniae* protein(s) or antibodies to a portion thereof in a host, e.g., by obtaining an antibody-containing sample from a relevant host, contacting the sample with expression product and detecting binding (for instance by having the product labeled); and, the antibodies generated by the aforementioned compositions are useful in diagnostic or detection kits or assays. Thus, the invention relates to varied compositions of matter and methods for use thereof.

BACKGROUND OF THE INVENTION

Streptococcus pneumoniae is an important cause of otitis media, meningitis, bacteremia and pneumonia. Despite the use of antibiotics and vaccines, the prevalence of pneumococcal infections has declined little over the last twenty-five years.

It is generally accepted that immunity to *Streptococcus pneumoniae* can be mediated by specific antibodies against the polysaccharide capsule of the pneumococcus. However, neonates and young children fail to make an immune response against polysaccharide antigens and can have repeated infections involving the same capsular serotype.

One approach to immunizing infants against a number of encapsulated bacteria is to conjugate the capsular polysaccharide antigens to protein to make them immunogenic. This approach has been successful, for example, with *Haemophilus influenzae b* (see U.S. Patent no. 4,496,538 to Gordon and U.S. Patent no. 4,673,574 to Anderson). However, there are over eighty known capsular serotypes of *S. pneumoniae* of which twenty-three account for most of the disease. For a pneumococcal polysaccharide-protein conjugate to be successful, the capsular types responsible for most pneumococcal infections would have to be made adequately immunogenic. This approach may be difficult, because the twenty-three polysaccharides included in the presently-available vaccine are not all adequately immunogenic, even in adults.

An alternative approach for protecting children, and also the elderly, from pneumococcal infection would be to identify protein antigens that could elicit protective immune responses. Such proteins may serve as a vaccine by themselves, may be used in conjunction with successful polysaccharide-protein conjugates, or as carriers for polysaccharides.

McDaniel et al. (I), J. Exp. Med. 160:386-397, 1984, relates to the production of hybridoma antibodies that recognize cell surface polypeptide(s) on *S. pneumoniae* and protection of mice from infection with certain strains of encapsulated pneumococci by such antibodies. This surface protein antigen has been termed "pneumococcal surface protein A" or PspA for short.

McDaniel et al. (II), Microbial Pathogenesis 1:519-531, 1986, relates to studies on the characterization of the PspA. Considerable diversity in the PspA molecule in different strains was found, as were differences in the epitopes recognized by different antibodies.

McDaniel et al. (III), J. Exp. Med. 165:381-394, 1987, relates to immunization of X-linked immunodeficient (XID) mice with non-encapsulated pneumococci expressing PspA, but not isogenic pneumococci lacking PspA, protects mice from subsequent fatal infection with pneumococci.

McDaniel et al. (IV), Infect. Immun., 59:222-228, 1991, relates to immunization of mice with a recombinant full length fragment of PspA that is able to elicit protection against pneumococcal strains of capsular types 6A and 3.

Crain et al, Infect.Immun., 56:3293-3299, 1990, relates to a rabbit antiserum that detects PspA in 100% (n = 95) of clinical and laboratory isolates of strains of *S. pneumoniae*. When reacted with seven monoclonal antibodies to PspA, fifty-seven *S. pneumoniae* isolates exhibited thirty-one different patterns of reactivity.

The PspA protein type is independent of capsular type. It would seem that genetic mutation or exchange in the environment has allowed for the development of a large pool of strains which are highly diverse with respect to capsule, PspA, and possibly other molecules with variable structures. Variability of PspA's from different strains also is evident in their molecular weights, which range from 67 to 99 kD. The observed differences are stably inherited and are not the result of protein degradation.

Immunization with a partially purified PspA from a recombinant λ gt11 clone, elicited protection against challenge with several *S. pneumoniae* strains representing different capsular and PspA types, as described in McDaniel et al. (IV), Infect. Immun. 59:222-228, 1991. Although clones expressing PspA were constructed according to that paper, the product was insoluble and isolation from cell fragments following lysis was not possible.

While the protein is variable in structure between different pneumococcal strains, numerous cross-reactions exist between all PspA's, suggesting that sufficient common epitopes may be present to allow a single PspA or at least a small number of PspA's to elicit protection against a large number of *S. pneumoniae* strains.

In addition to the published literature specifically referred to above, the inventors, in conjunction with co-workers, have published further details concerning PspA's, as follows:

1. Abstracts of 89th Annual Meeting of the American Society for Microbiology, p. 125, item D-257, May 1989;
2. Abstracts of 90th Annual Meeting of the American Society for Microbiology, p. 98, item D-106, May 1990;
3. Abstracts of 3rd International ASM Conference on Streptococcal Genetics, p. 11, item 12, June 1990;
4. Talkington et al, Infect. Immun. 59:1285-1289, 1991;
5. Yother et al (I), J. Bacteriol. 174:601-609, 1992; and
6. Yother et al (II), J. Bacteriol. 174:610-618, 1992.
7. McDaniel et al (V), Microbiol. Pathogenesis, 13:261-268.

It would be useful to provide PspA or fragments thereof in compositions, including PspA's or fragments from varying strains in such compositions, to provide antigenic, immunological or vaccine compositions; and, it is even further useful to show that the various strains can be grouped or typed, thereby providing a basis for cross-reactivities of PspA's or fragments thereof, and thus providing a means for determining which strains to represent in such compositions (as well as how to test for, detect or diagnose one strain from another).

Further, it would be advantageous to provide a *pspA* - like gene or a *pspC* gene in certain strains, as well as primers (oligonucleotides) for identification of such a gene, as well as of conserved regions in that gene and in *pspA*; for instance, for detecting, determining, isolating, or diagnosing strains of *S. pneumonia*. These uses and advantages, it is believed, have not heretofore been provided in the art.

OBJECTS AND SUMMARY OF THE INVENTION

The invention provides an isolated amino acid molecule comprising residues 1 to 115, 1 to 260, 192 to 588, 192 to 299, or residues 192 to 260 of pneumococcal surface protein A of *Streptococcus pneumoniae*.

The invention further provides an isolated DNA molecule comprising a fragment of a pneumococcal surface protein A gene of *Streptococcus pneumoniae* encoding the isolated amino acid molecule.

The invention also provides PCR primers or hybridization probes comprising the isolated DNA molecule.

The invention additionally provides an antigenic, vaccine or immunological composition comprising the amino acid molecule.

The invention includes an isolated DNA molecule comprising nucleotides 1 to 26, 1967 to 1990, 161 to 187, 1093 to 1117, or 1312 to 1331 or 1333 to 1355 of a pneumococcal surface protein A gene of *Streptococcus pneumoniae*. The DNA molecule can be used as a PCR primer or hybridization probe; and therefore the invention comprehends a PCR primer or hybridization probe comprising the isolated DNA molecule.

The invention also includes an isolated DNA molecule comprising a fragment having homology with a portion of a pneumococcal surface protein A gene of *Streptococcus pneumoniae*. The DNA preferably is the following (which include the portion having homology and restriction sites, and selection of other restriction sites or sequences for such DNA is within the ambit of the skilled artisan from this disclosure):

CCGGATCCAGCTCCTGCACCAAAAAC;
GCGCGTCGACGGCTTAAACCCATTCACCATTGG;
CCGGATCCTGAGCCAGAGCAGTTGGCTG;
CCGGATCCGCTCAAAGAGATTGATGAGTCTG;
GCGGATCCCGTAGCCAGTCAGTCTAAAGCTG;
CTGAGTCGACTGGAGTTTCTGGAGCTGGAGC;
CCGGATCCAGCTCCAGCTCCAGAAACTCCAG;
GCGGATCCTTGACCAATATTTACGGAGGAGGC;
GTTTTTGGTGCAGGAGCTGG;
GCTATGGGCTACAGGTTG;
CCACCTGTAGCCATAGC;
CCGCATCCAGCGTGCCTATCTTAGGGGCTGGTT; and
GCAAGCTTATGATATAGAAATTTGTAAC

(thus, the invention broadly comprehends DNA homologous to portions of *pspA*; preferably further including restriction sequences).

These DNA molecules can be used as PCR primers or probes; and thus, the invention comprehends a primer or probe comprising and of these molecules.

The invention further still provides PCR probe(s) which distinguishes between *pspA* and *pspA*-like nucleotide sequence, as well as PCR probe(s) which hybridizes to both *pspA* and *pspA*-like nucleotide sequences.

Additionally, the invention includes a PspA extract prepared by a process comprising: growing pneumococci in a first medium containing choline chloride, eluting live pneumococci with a choline chloride containing salt solution, and growing the pneumococci in a second medium containing an alkanolamine and substantially no choline; as well as a PspA extract prepared by that process and further comprising purifying PspA by isolation on a choline-Sepharose affinity column. These processes are also included in the invention.

An immunological composition comprising thses extracts is comprehended by the invention, as well as an immunological composition comprising the full length PspA.

A method for enhancing the immunogenicity of a PspA-containing immunological composition comprising, in said composition, the C-terminal portion of PspA, is additionally comprehended, as well.

An immunological composition comprising at least two PspAs. The latter immunological composition can have the PspAs from different groups or families; the groups or families can be based on RFLP or sequence studies (see, e.g., Fig. 13).

Further, the invention provides an isolated amino acid molecule comprising pneumococcal surface protein C, PspC, of *Streptococcus pneumoniae* having an alpha-helical, proline rich and repeat regions, an isolated DNA molecule comprising a pneumcoccal surface protein C gene encoding the aforementioned PspC, and primers and hybrization probes consisting essentially of the isolated DNA molecule.

Still further, an isolated amino acid molecule comprising pneumococcal surface protein C, PspC, of *Streptococcus pneumoniae* is provided, having an alpha-helical, proline rich and repeat regions, having substantial homology with a protection eliciting region of PspA, and an isolated DNA molecule comprising a pneumcoccal surface protein C gene encoding the aforementioned PspC, and primers and hybrization probes consisting essentially of the isolated DNA molecule are provided by the present invention.

Additionally, the present invention provides immunological compositions comprising PspC.

These and other embodiments are disclosed or are obvious from the following detailed description.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A and 1B show: Evaluation of digested plasmid constructs. Fig. 1A: 1% agarose gel electrophoresis of plasmids isolated from transformed *E.*

coli BL21(DE3) strains stained with ethidium bromide. Lane 1: 1 kb DNA ladder (sizes noted in kb), lane 2: pRCT125; lane 3: pRC105, lane 4: DBL5 pspA insert, lane 5: pRCT113, lane 6: BG9739 pspA insert, lane 7: pRCT117, and lane 8: L81905 pspA insert. Fig. 1B: Corresponding Southern blot of gel in Fig. 1A probed with full-length Rx1 pspA and hybridization detected as described in Example 1. The arrow indicates the 1.2 kb pspA digested inserts from plasmid constructs and the PCR-amplified pspA fragments from the pneumococcal donor strains used in cloning.

Figure 2 shows: Evaluation of strain RCT105 cell fractions containing truncated DBL5 PspA. Proteins from *E. coli* cell fractions were resolved by 10% SDS-PAGE, transferred to NC, and probed with MAb XiR278. Lane 1: molecular weight markers (noted in kDa), lane 2: full-length, native DBL5 PspA, lane 3: uninduced cells, lanes 4-6: induced cells; 1 hr, 2 hr, and 3 hr of IPTG induction respectively, lane 7: periplasmic proteins, lane 8: cytoplasmic proteins, and lane 9: insoluble cell wall/membrane material.

Figure 3 shows: SDS-PAGE of R36A PspA (80 ng) column isolated from CDM-ET and an equal volume of an equivalent WG44.1 prep. Identical gels are shown stained with Bio-Rad silver kit (A) or immunoblotted with PspA MAb XiR278(B). The PspA isolated from R36A shows the characteristic monomer (84 kDa) and dimer bands.

Figure 4 shows: Cell lysates of pneumococcal isolates MC27 and MC28 were subjected to SDS-PAGE and transferred to nitrocellulose for Western blotting with seven MAb to PspA. 7D2 detected a protein of 82 kDa in each isolate and XiR278 and 2A4 detected a protein of 190 kDa in each isolate. MAb Xi64, Xi126, 1A4 and SR4W4 were not reactive. Strains MC25 and MC26 yielded identical results.

Figure 5 (Figs. 5A and 5B) shows: Southern blot of *Hind* III digest of MC25-MC28 chromosomal DNA developed at a stringency greater than 95 percent. A digest of Rx1 DNA was used as a comparison. The blot was probed with LSM*pspA*13/2, a full length Rx1 probe (Fig. 5) and LSM*pspA*12/6 a 5′ probe of Rx1 *pspA* (Fig. 5). The same concentration of Rx1 DNA was used in both panels, but the concentrations of MC25-MC28 DNA in Fig. 5B were half that used in Fig. 5A to avoid detection of partial digests.

Figure 6 shows: RFLP of amplified *pspA*. *PspA* from MC25 was amplified by PCR using 5′ and 3′ primers for *pspA* (LSM13 and LSM, respectively). The amplified DNA was digested with individual restriction endonucleases prior to electrophoresis and staining with ethidium bromide. Lane 1 *Bcl*I, Lane 2 *BAM*HI, Lane 3 *Bst*NI, Lane 4 *Pst*I, Lane 5 *Sac*I, Lane 6 *Eco*RI, Lane 7 *Sma*I, Lane 8 *Kpn*I.

Figure 7 shows: A depiction of PspA showing the relative location and orientation of the oligonucleotides.

Figure 8 shows: Derivatives of the *S. pneumoniae* D39-Rx1 family.

Figures 9 to 10 show: Electrophoresis of *pspA* or amplified *pspA* product with *Hha*I (Fig. 9), *Sau*3AI (Fig. 10).

Figure 11 shows: RFLP pattern of two isolates from six families.

Figure 12 shows: RFLP pattern of two isolates from six families (using products from amplification with SKH2 and LSM13).

Figure 13 shows: Sequence primarily in the N-terminal half of PspA.

Figure 14 shows: Cell lysates of pneumococcal isolates MC27 and MC28, subjected to SDS-PAGE and Western blotting with seven MAbs to PspA; 7D2 detected a protein of 82 kDa in each isolate, and Xi278 and 2A4 detected a protein of 190 kDa in each isolate; MAbs Xi64, Xi126, 1A4 and SR4W4 were not reactive; strains MC25 and MC26 yielded identical results (not shown).

Figure 15A and 15B show: a Southern blot of *Hin*d III digest of MC25-28 chromosomal DNA, using a digest of Rx1 DNA as a comparison; the blot was probed with LSM*pspA*13/2, a full length Rx1 probe (A), and LSM*pspA*12/6, a 5′ probe of Rx1 *pspA* (B); the same concentration of Rx1 DNA was used in both panels, but the concentrations of MC25-28 DNA in B were half that used in A to avoid detection of partial digests.

Figures 15C and 15D show: the nucleotide sequences of primers LSM13, LSM2, LSM12 and LSM6, and that of probes LSM*pspA*13/2 and LSM*pspA*12/6.

Figure 16 shows: RFLP of amplified *pspA*, wherein PspA from MC25 was amplified by PCR using 5′ and 3′ primers for *pspA* (LSM13 and LSM2, respectively); the amplified DNA was digested with individual restriction endonucleases prior to electrophoresis and staining with ethidium bromide; *Bcl* I was used in lane 1; *BamH* I was used in lane 2; *BstN* I was used in lane 3; *Pst* I was used in lane 4; *Sac* I was used in lane 5; *EcoR* I was used in lane 6; *Sma* I was used in lane 7; and *Kpn* I was used in lane 8.

Figure 17 shows: position and orientation of oligonucleotides relative to domains encoded by *pspA*; numbers along the bottom of the Figure represent amino acids in the mature PspA polypeptide from strain Rx1, and arrows represent the relative position (not to scale) and orientation of oligonucleotides.

Figure 18 shows: a restriction map of the pZero vector.

Figure 19 shows: the nucleotide sequences of SKH2, LSM13, N192 and C588.

Figure 20 shows: a comparison of the structural motifs of PspA and PspC; PspA has a smaller alpha-helical region, and does not contain the direct repeats within the alpha-helix (indicated by the dashed lines); the alpha-helical regions which are homologous between PspA and PspC are indicated by the dashed lines); the alpha-helical regions which are homologous between PspA and PspC are indicated by the striped pattern; and PCR primers are indicated by the arrows.

Figure 21 shows: the amino acid and nucleotide sequence of PspC, wherein the putative -10 and -35 regions are underlined, and the ribosomal binding site is in lower case.

Figure 22 shows: the Bestfit analysis of PspA and PspC; percent identity is 69% and percent similarity is 77%; amino acids of PspA are one the bottom line (1-588) and amino acids of PspC are on the top line (249-891), and a dashed line indicated identity.

Figure 23 shows: the coiled coil motif of the alpha-helix of PspC; amino acids that are not in the coiled coil motif are in the right column.

Figure 24 shows: a matrix plot comparison of the repeat regions of the alpha-helical region of PspC.

Figure 25 shows: the sequence of the alpha helical and proline regions of LXS532 (PspC.D39).

Figure 26 shows: a comparison of nucleotides of *pspA*.Rx1 to *pspC*.D39.

Figure 27 shows: a BESTFIT analysis of *pspC*.EF6797 and *pspC*.D39.

Figure 28 shows: the amino acid comparison of PspC of EF6797 and D39.

Figure 29 shows: the amino acid comparison of PspC.D39 and PspA.Rx1.

DETAILED DESCRIPTION

Knowledge of and familiarity with the applications incorporated herein by reference is assumed; and, those applications disclose the sequence of *pspA* as well as certain portions thereof, and PspA and compositions containing PspA.

As discussed above and in the following Examples, the invention relates to truncated PspA, e.g., PspA C-terminal to position 192 such as a.a. 192-588 ("BC100") 192-299 and 192-260 of PspA eliciting cross-protection, as well as to DNA encoding such truncated PspA (which amplify the coding for these amino acid regions homologous to most PspAs).

The invention further relates to a *pspA*-like gene, or a *pspC* gene and portions thereof (e.g., probes, primers) which can hybridize thereto and/or amplify that gene, as well as to DNA molecules which hybridize to *pspA*, so that one can, by hybridization assay and/or amplification, ascertain the presence of a particular pneumococcal strain; and, the invention provides that a PspC can be produced by the *pspA*-like or *pspC* sequence (which PspC can be used like PspA).

Indeed, the invention further relates to oligonucleotide probes and/or primers which react with *pspA* and/or *pspC* of many, if not all, strains, so as to permit identification, detection or diagnosis of any pneumococcal strain, as well as to expression products of such probes and/or primers, which can provide cross-reactive epitopes of interest.

The repeat region of *pspA* and/or *pspC* is highly conserved such that the present invention provides oligonucleotide probes or primers to this region reactive with most, if not all strains, thereby providing diagnostic assays and a means for identifying epitopes of interest.

The invention demonstrates that the *pspC* gene is homologous to the *pspA* gene in the leader sequence, first portion of the proline-rich region and in the repeat region; but, these genes differ in the second portion of their proline-rich regions and at the very 3′ end of the gene encoding the 17 amino acid tail of PspA. The product of the *pspC* gene is expected to lack a C-terminal tail, suggesting different anchoring than PspA. Drug interference with functions such as surface binding of the coding for repeat regions of *pspA* and the *pspC* genes, or with the repeat regions of the expression products, is therefore a target for intervention of pneumococcal infection.

Further still, the invention provides evidence of additional *pspA* homologous sequences, in addition to *pspA* and the *pspC* sequence. The invention, as mentioned above, includes oligonucleotide probes or primers which distinguish between *pspA* and the *pspC* sequence, e.g., LSM1 and LSM2, useful for diagnostic detecting, or isolating purposes; and LSM1 and LSM10 or LSM1 and LSM7 which amplify a portion of the *pspC* gene, particularly the portion of that gene which encodes an antigenic, immunological or protective protein.

The invention further relates to a method for the isolation of native PspA by growth of pneumococci medium containing high concentrations of(about 0.9% to about 1.4%, preferably 1.2%) choline chloride, elation of live pneumococci with a salt solution containing choline chloride, e.g., about 1% about 3%, preferably 2% choline chloride, and growth of pneumococci in medium in which the choline in the medium has been almost or substantially completely replaced with a lower alkanolamine, e.g., C_1-C_6, preferably C_2 alkanolamine, i.e., preferably C_2 alkanolamine, i.e., preferably ethanolamine (e.g., 0.0000005% to 0.0000015%, preferably 0.000001% choline chloride plus 0.02% to 0.04% alkanolamine (ethanolamine), preferably 0.03%). PspA from such pneumococci is then preferably isolated from a choline-sepharose affinity column, thereby providing highly purified PspA. Such isolated and/or purified PspA is highly immunogenic and is useful in antigenic, immunological or vaccine composition.

Indeed, the growth media of the pneumococci grown in the presence of the alkanolamine (rather than choline) contains PspA and is itself highly immunogenic and therefore useful as an antigenic, immunological or vaccine composition; and, is rather inexpensive to produce. Per microgram of PspA, the PspA in the alkanolamine medium is much more protective than PspA isolated by other means, e.g., from extracts. Perhaps, without wishing to necessarily be bound by any one particular theory, there is a synergistic effect upon PspA by the other components present prior to isolation, or simply PspA is more protective (more antigenic) prior to isolation and/or purification (implying a possibility of some loss of activity from the step of isolation and/or purification).

The invention further relates to the N-terminal 115 amino acids of PspA, which is useful for compositions comprising an epitope of interest, immunological or vaccine compositions, as well as the DNA coding therefor, which is useful in preparing these N-terminal amino acids by recombination, or for use as probes and/or primers for hybridization and/or amplification for identification, detection or diagnosis purposes.

The invention further demonstrates that there is a grouping among the *pspA* RFLP families. This provides a method of identifying families of different PspAs based on RFLP pattern of *pspAs*, as well as a means for obtaining diversity of PspAs in an antigenic, immunological or vaccine composition; and, a method of characterizing clonotypes of PspA based on RFLP patterns of PspA. And, the invention thus provides oligonucleotides which permit amplification of most, e.g., a majority, if not all of *S. pneumoniae* and thereby permit RFLP analysis of a majority, if not all, *S. pneumoniae*.

The invention also provides PspC, having an approximate molecular weight of 105 kD, with an estimated pI of 6.09, and comprising an alpha-helical region, followed by a proline-rich domain and repeat region. A major cross-protective region of PspA comprises the C-terminal third of the alpha-helical region (between residues 192 and 260 of PspA), which region accounts for the binding of 4 of 5 cross-protective MAb, and PspA fragments comprising this region can elicit cross-protective immunity in mice. Homology between PspC and PspA begins at amino acid 148 of PspA, thus including the region from 192 to 299, and including the entire PspC sequence C-terminal of amino acid 486. Due to the substantial sequence homology between PspA and PspC in a region comprising the epitopes of interest, known to be protection eliciting, PspC is likely to comprise epitopes of interest similar to those found in PspA. Antibodies specific for this region of PspA, i.e., between amino acids 148 and 299, should cross-react with PspC, and thus afford protection by reacting with PspC and PspA. Similarly, immunization with PspC would be expected to elicit antibodies cross-protective against PspA.

An epitope of interest is an antigen or immunogen or immunologically active fragment thereof from a pathogen or toxin of veterinary or human interest.

The present invention provides an immunogenic, immunological or vaccine composition containing the pneumococcal epitope of interest, and a pharmaceutically acceptable carrier or diluent. An immunological composition containing the pneumococcal epitope of interest, elicits an immunological response - local or systemic. The response can, but need not be, protective. Am immunogenic composition containing the pneumococcal epitope of interest, likewise elicits a local or systemic immunological response which can, but need not be, protective. A vaccine composition elicits a local or systemic protective response. Accordingly, the terms "immunological composition" and "immunogenic composition" include a "vaccine composition" (as the two former terms can be protective compositions).

The invention therefore also provides a method of inducing an immunological response in a host mammal comprising administering to the host an immunogenic, immunological or vaccine composition comprising the pneumococcal epitope of interest, and a pharmaceutically acceptable carrier or diluent.

The DNA encoding the pneumococcal epitope of interest can be DNA which codes for full length PspA, PspC, or fragments thereof. A sequence which codes for a fragment of PspA or PspC can encode that portion of PspA or PspC which contains an epitope of interest, such as a protection-eliciting epitope of the protein.

Regions of PspA and PspC have been identified from the Rx1 strain of *S. pneumoniae* which not only contain protection-eliciting epitopes, but are also sufficiently cross-reactive with other PspAs from other *S. pneumoniae* strains so as to be suitable candidates for the region of PspA to be incorporated into a vaccine, immunological or immunogenic composition. Epitopic regions of PspA include residues 1 to 115, 1 to 314, 192 to 260 and 192 to 588. DNA encoding fragments of PspA can comprise DNA which codes for the aforementioned epitopic regions of PspA; or it can comprise DNA encoding overlapping fragments of PspA, e.g., fragment 192 to 588 includes 192 to 260, and fragment 1 to 314 includes 1 to 115 and 192 to 260.

As to epitopes of interest, one skilled in the art can determine an epitope of immunodominant region of a peptide or polypeptide and ergo the coding DNA therefor from the knowledge of the amino acid and corresponding DNA sequences of the peptide or polypeptide, as well as from the nature of particular amino acids (e.g., size, charge, etc.) and the codon dictionary, without undue experimentation.

A general method for determining which portions of a protein to use in an immunological composition focuses on the size and sequence of the antigen of interest. "In general, large proteins, because they have more potential determinants are better antigens than small ones. The more foreign an antigen, that is the less similar to self configurations which induce tolerance, the more effective it is in provoking an immune response." Ivan Roitt, Essential Immunology, 1988.

As to size, the skilled artisan can maximize the size of the protein encoded by the DNA sequence to be inserted into the viral vector (keeping in mind the packaging limitations of the vector). To minimize the DNA inserted while maximizing the size of the protein expressed, the DNA sequence can exclude introns (regions of a gene which are transcribed but which are subsequently excised from the primary RNA transcript).

At a minimum, the DNA sequence can code for a peptide at least 8 or 9 amino acids long. This is the minimum length that a peptide needs to be in order to stimulate a CD4+ T cell response (which recognizes virus infected cells or cancerous cells). A minimum peptide length of 13 to 25 amino acids is useful to stimulate a CD8+ T cell response (which recognizes special antigen presenting cells which have engulfed the pathogen). See Kendrew, *supra*. However, as these are minimum lengths, these peptides are likely to generate an immunological response, i.e., an antibody or T cell response; but, for a protective response (as from a vaccine composition), a longer peptide is preferred.

With respect to the sequence, the DNA sequence preferably encodes at least regions of the peptide that generate an antibody response or a T cell response. One method to determine T and B cell epitopes involves epitope mapping. The protein of interest "is fragmented into overlapping peptides with proteolytic enzymes. The individual peptides are then tested for their ability to bind to an antibody elicited by the native protein or to induce T cell or B cell activation. This approach has been particularly useful in mapping T-cell epitopes since the T cell recognizes short linear peptides complexed with MHC molecules. The method is less effective for determining B-cell epitopes" since B cell epitopes are often not linear amino acid sequence but rather result from the tertiary structure of the folded three dimensional protein. Janis Kuby, <u>Immunology</u>, (1992) pp. 79-80.

Another method for determining an epitope of interest is to choose the regions of the protein that are hydrophilic. Hydrophilic residues are often on the surface of the protein and therefore often the regions of the protein which are accessible to the antibody. Janis Kuby, <u>Immunology</u>, (1992) P. 81.

Yet another method for determining an epitope of interest is to perform an X-ray cyrstallographic analysis of the antigen (full length)-antibody complex. Janis Kuby, <u>Immunology</u>, (1992) p. 80.

Still another method for choosing an epitope of interest which can generate a T cell response is to identify from the protein sequence potential HLA anchor binding motifs which are peptide sequences which are known to be likely to bind to the MHC molecule.

The peptide which is a putative epitope, to generate a T cell response, should be presented in a MHC complex. The peptide preferably contains appropriate anchor motifs for binding to the MHC molecules, and should bind with high enough affinity to generate an immune response. Factors which can be considered are: the HLA type of the patient (vertebrate, animal or human) expected to be immunized, the sequence of the protein, the presence of appropriate anchor motifs and the occurance of the peptide sequence in other vital cells.

An immune response is generated, in general, as follows: T cells recognize proteins only when the protein has been cleaved into smaller peptides and is presented in a complex called the "major histocompatability complex MHC" located on another cell's surface. There are two classes of MHC complexes - class I and class II, and each class is made up of many different alleles. Different patients have different types of MHC complex alleles; they are said to have a 'different HLA type'.

Class I MHC complexes are found on virtually every cell and present peptides from proteins produced inside the cell. Thus, Class I MHC complexes are useful for killing cells which when infected by viruses or which have become cancerous and as the result of expression of an oncogene. T cells which have a protein called CD4 on their surface, bind to the MHC class I cells and secrete lymphokines. The lymphokines stimulate a response; cells arrive and kill the viral infected cell.

Class II MHC complexes are found only on antigen- presenting cells and are used to present peptides from circulating pathogens which have been endocytosed by the antigen- presenting cells. T cells which have a protein called CD8 bind to the MHC class II cells and kill the cell by exocytosis of lytic granules.

Some guidelines in determining whether a protein is an epitopes of interest which will stimulate a T cell response, include: Peptide length - the peptide should be at least 8 or 9 amino acids long to fit into the MHC class I complex and at least 13-25 amino acids long to fit into a class II MHC complex. This length is a minimum for the peptide to bind to the MHC complex. It is preferred for the peptides to be longer than these lengths because cells may cut the expressed peptides. The peptide should contain an appropriate anchor motif which will enable it to bind to the various class I or class II molecules with high enough specificity to generate an immune response (See Bocchia, M. et al, Specific Binding of Leukemia Oncogene Fusion Protein Peptides to HLA Class I Molecules, Blood 85:2680-2684; Englehard, VH, Structure of peptides associated with class I and class II MHC molecules Ann. Rev. Immunol. 12:181 (1994)). This can be done, without undue experimentation, by comparing the sequence of the protein of interest with published structures of peptides associated with the MHC molecules. Protein epitopes recognized by T cell receptors are peptides generated by enzymatic degradation of the protein molecule and are prestnted on the cell surface in association with class I or class II MHC molecules.

Further, the skilled artisan can ascertain an epitope of interest by comparing the protein sequence with sequences listed in the protein data base. Regions of the protein which share little or no homology are better choices for being an epitope of that protein and are therefore useful in a vaccine or immunological composition. Regions which share great homology with widely found sequences present in vital cells should be avoided.

Even further, another method is simply to generate or express portions of a protein of interest, generate monoclonal antibodies to those portions of the protein of interest, and then ascertain whether those antibodies inhibit growth *in vitro* of the pathogen from which the from which the protein was derived. The skilled artisan can use the other guidelines set forth in this disclosure and in the art for generating or expressing portions of a protein of interest for analysis as to whether antibodies thereto inhibit growth *in vitro*. For example, the skilled artisan can generate portions of a protein of interest by: selecting 8 to 9 or 13 to 25 amino acid length portions of the protein, selecting hydrophilic regions, selecting portions shown to bind from X-ray data of the antigen (full length)-antibody complex, selecting regions which differ in sequence from other proteins, selecting potential HLA anchor binding motifs, or any combination of these methods or other methods known in the art.

Epitopes recognized by antibodies are expressed on the surface of a protein. To determine the regions of a protein most likely to stimulate an antibody response one skilled in the art can preferably perform an epitope map, using the general methods described above, or other mapping methods known in the art.

As can be seen from the foregoing, without undue experimentation, from this disclosure and the knowledge in the art, the skilled artisan can ascertain the amino acid and corresponding DNA sequence of an epitope of interest for obtaining a T cell, B cell and/or antibody response. In addition, reference is made to Gefter et al., U.S. Patent No. 5,019,384, issued May 28, 1991, and the documents it cites, incorporated herein by reference (Note especially the "Relevant Literature" section of this patent, and column 13 of this patent which discloses that: "A large number of epitopes have been defined for a wide variety of organisms of interest. Of particular interest are those epitopes to which neutralizing antibodies are directed. Disclosures of such epitopes are in many of the references cited in the Relevant Literature section.")

Further, the invention demonstrates that more than one serologically complementary PspA molecule can be in an antigenic, immunological or vaccine composition, so as to elicit better response, e.g., protection, for instance, against a variety of strains of pneumococci; and, the invention provides a system of selecting PspAs for a multivalent composition which includes cross-protection evaluation so as to provide a maximally efficacious composition.

The determination of the amount of antigen, e.g., PspA or truncated portion thereof and optional adjuvant in the inventive compositions and the preparation of those compositions can be in accordance with standard techniques well known to those skilled in the pharmaceutical or veterinary arts. In particular, the amount of antigen and adjuvant in the inventive compositions and the dosages administered are determined by techniques well known to those skilled in the medical or veterinary arts taking into consideration such factors as the particular antigen, the adjuvant (if present), the age, sex, weight, species and condition of the particular patient, and the route of administration. For instance, dosages of particular PspA antigens for suitable hosts in which an immunological response is desired, can be readily ascertained by those skilled in the art from this disclosure (see, e.g., the Examples), as is the amount of any adjuvant typically administered therewith. Thus, the skilled artisan can readily determine the amount of antigen and optional adjuvant in compositions and to be administered in methods of the invention. Typically, an adjuvant is commonly used as 0.001 to 50 wt% solution in phosphate buffered saline, and the antigen is present on the order of micrograms to milligrams, such as about 0.0001 to about 5 wt%, preferably about 0.0001 to about 1 wt%, most preferably about 0.0001 to about 0.05 wt% (see, e.g., Examples below or in applications cited herein).

Typically, however, the antigen is present in an amount on the order of micrograms to milligrams, or, about 0.001 to about 20 wt%, preferably about 0.01 to about 10 wt%, and most preferably about 0.05 to about 5 wt% (see, e.g., Examples below).

Of course, for any composition to be administered to an animal or human, including the components thereof, and for any particular method of administration, it is preferred to determine therefor: toxicity, such as by determining the lethal dose (LD) and LD_{50} in a suitable animal model e.g., rodent such as mouse; and, the dosage of the composition(s), concentration of components therein and timing of administering the composition(s), which elicit a suitable immunological response, such as by titrations of sera and analysis thereof for antibodies or antigens, e.g., by ELISA and/or RFFIT analysis. Such determinations do not require undue experimentation from the knowledge of the skilled artisan, this disclosure and the documents cited herein. And, the time for sequential administrations can be ascertained without undue experimentation.

Examples of compositions of the invention include liquid preparations for orifice, e.g., oral, nasal, anal, vaginal, peroral, intragastric, mucosal (e.g., perlingual, alveolar, gingival, olfactory or respiratory mucosa) etc., administration such as suspensions, syrups or elixirs; and, preparations for parenteral, subcutaneous, intradermal, intramuscular or intravenous administration (e.g., injectable administration), such as sterile suspensions or emulsions. Such compositions may be in admixture with a suitable carrier, diluent, or excipient such as sterile water, physiological saline, glucose or the like. The compositions can also be lyophilized. The compositions can contain auxiliary substances such as wetting or emulsifying agents, pH buffering agents, gelling or viscosity enhancing additives, preservatives, flavoring agents, colors, and the like, depending upon the route of administration and the preparation desired. Standard texts, such as "REMINGTON'S PHARMACEUTICAL SCIENCE", 17th edition, 1985, incorporated herein by reference, may be consulted to prepare suitable preparations, without undue experimentation.

Compositions of the invention, are conveniently provided as liquid preparations, e.g., isotonic aqueous solutions, suspensions, emulsions or viscous compositions which may be buffered to a selected pH. If digestive tract absorption is preferred, compositions of the invention can be in the "solid" form of pills, tablets, capsules, caplets and the like, including "solid" preparations which are time-released or which have a liquid filling, e.g., gelatin covered liquid, whereby the gelatin is dissolved in the stomach for delivery to the gut. If nasal or respiratory (mucosal) administration is desired, compositions may be in a form and dispensed by a squeeze spray dispenser, pump dispenser or aerosol dispenser. Aerosols are usually under pressure by means of a hydrocarbon. Pump dispensers can preferably dispense a metered dose or, a dose having a particular particle size.

Compositions of the invention can contain pharmaceutically acceptable flavors and/or colors for rendering them more appealing, especially if they are administered orally. The viscous compositions may be in the form of gels, lotions, ointments, creams and the like and will typically contain a sufficient amount of a thickening agent so that the viscosity is from about 2500 to 6500 cps, although more viscous compositions, even up to 10,000 cps may be employed. Viscous compositions have a viscosity preferably of 2500 to 5000 cps, since above that range they become more difficult to administer. However, above that range, the compositions can approach solid or gelatin forms which are then easily administered as a swallowed pill for oral ingestion.

Liquid preparations are normally easier to prepare than gels, other viscous compositions, and solid compositions. Additionally, liquid compositions are somewhat more convenient to administer, especially by injection or orally, to animals, children, particularly small children, and others who may have difficulty swallowing a pill, tablet, capsule or the like, or in multi-dose situations. Viscous compositions, on the other hand, can be formulated within the appropriate viscosity range to provide longer contact periods with mucosa, such as the lining of the stomach or nasal mucosa.

Obviously, the choice of suitable carriers and other additives will depend on the exact route of administration and the nature of the particular dosage form, e.g., liquid dosage form [e.g., whether the composition is to be formulated into a solution, a suspension, gel or another liquid form], or solid dosage form [e.g., whether the composition is to be formulated into a pill, tablet, capsule, caplet, time release form or liquid-filled form].

Solutions, suspensions and gels, normally contain a major amount of water (preferably purified water) in addition to the antigen, lipoprotein and optional adjuvant. Minor amounts of other ingredients such as pH adjusters (e.g., a base such as NaOH), emulsifiers or dispersing agents, buffering agents, preservatives, wetting agents, jelling agents, (e.g., methylcellulose), colors and/or flavors may also be present. The compositions can be isotonic, i.e., it can have the same osmotic pressure as blood and lacrimal fluid.

The desired isotonicity of the compositions of this invention may be accomplished using sodium chloride, or other pharmaceutically acceptable agents such as dextrose, boric acid, sodium tartrate, propylene glycol or other inorganic or organic solutes. Sodium chloride is preferred particularly for buffers containing sodium ions.

Viscosity of the compositions may be maintained at the selected level using a pharmaceutically acceptable thickening agent. Methylcellulose is preferred because it is readily and economically available and is easy to work with. Other suitable thickening agents include, for example, xanthan gum, carboxymethyl cellulose, hydroxypropyl cellulose, carbomer, and the like. The preferred concentration of the thickener will depend upon the agent selected. The important point is to use an amount which will achieve the selected viscosity. Viscous compositions are normally prepared from solutions by the addition of such thickening agents.

A pharmaceutically acceptable preservative can be employed to increase the shelf-life of the compositions. Benzyl alcohol may be suitable, although a variety of preservatives including, for example, parabens, thimerosal, chlorobutanol, or benzalkonium chloride may also be employed. A suitable concentration of the preservative will be from 0.02% to 2% based on the total weight although there may be appreciable variation depending upon the agent selected.

Those skilled in the art will recognize that the components of the compositions must be selected to be chemically inert with respect to the PspA antigen and optional adjuvant. This will present no problem to those skilled in chemical and pharmaceutical principles, or problems can be readily avoided by reference to standard texts or by simple experiments (not involving undue experimentation), from this disclosure and the documents cited herein.

The immunologically effective compositions of this invention are prepared by mixing the ingredients following generally accepted procedures. For example the selected components may be simply mixed in a blender, or other standard device to produce a concentrated mixture which may then be adjusted to the final concentration and viscosity by the addition of water or thickening agent and possibly a buffer to control pH or an additional solute to control tonicity. Generally the pH may be from about 3 to 7.5. Compositions can be administered in dosages and by techniques well known to those skilled in the medical and veterinary arts taking into consideration such factors as the age, sex, weight, and condition of the particular patient or animal, and the composition form used for administration (e.g., solid vs. liquid). Dosages for humans or other mammals can be determined without undue experimentation by the skilled artisan, from this disclosure, the documents cited herein, the Examples below (e.g., from the Examples involving mice).

Suitable regimes for initial administration and booster doses or for sequential administrations also are variable, may include an initial administration followed by subsequent administrations; but nonetheless, may be ascertained by the skilled artisan, from this disclosure, the documents cited herein, and the Examples below.

PCR techniques for amplifying sample DNA for diagnostic detection or assay methods are known from the art cited herein and the documents cited herein (see Examples), as are hybridization techniques for such methods. And, without undue experimentation, the skilled artisan can use gene products and antibodies therefrom in diagnostic, detection or assay methods by procedures known in the art.

The following Examples are provided for illustration and are not to be considered a limitation of the invention.

EXAMPLES

EXAMPLE 1 - TRUNCATED STREPTOCOCCUS PNEUMONIAE PspA MOLECULES ELICIT CROSS-PROTECTIVE IMMUNITY AGAINST PNEUMOCOCCAL CHALLENGE

Since the isolation of *S. pneumoniae* from human saliva in 1881 and its subsequent connection with lobar pneumonia two years later, human disease resulting from pneumococcal infection has been associated with a significant degree of morbidity and mortality. A recent survey of urgently needed vaccines in the developing and developed world places an improved pneumococcal vaccine among the top three vaccine priorities of industrialized countries. The currently licensed vaccine is a 23-valent composition of pneumococcal capsular polysaccharides that is only about 60% effective in the elderly and due to poor efficacy is not recommended for use in children below two years of age. Furthermore the growing frequency of multi-drug resistant strains of *S. pneumoniae* being isolated accentuates the need for a more effective vaccine to prevent pneumococcal infections.

The immunogenic nature of proteins makes them prime targets for new vaccine strategies. Pneumococcal molecules being investigated as potential protein vaccine candidates include pneumolysis, neuraminidase, autolysin and PspA. All of these proteins are capable of eliciting immunity in mice resulting in extension of life and protection against death with challenge doses near the LD_{50}. PspA is unique among these macromolecules in that is can elicit antibodies in animals that protect against inoculums 100-fold greater than the LD_{50}.

PspA is a surface-exposed protein with an apparent molecular weight of 67-99 kDa that is expressed by all clinically relevant *S. pneumoniae* strains examined to date. Though PspAs from different pneumococcal strains are serologically variable, many PspA antibodies exhibit cross-reactivities with PspAs from unrelated strains. Upon active immunization with PspA, mice generate PspA antibodies that protect against subsequent challenge with diverse strains of *S. pneumoniae*. The immunogenic and protection-eliciting properties of PspA suggest that it may be a good candidate molecule for a protein-based pneumococcal vaccine.

Four distinct domains of PspA have been identified based on DNA sequence. They include a N-terminal highly charged alpha-helical region, a proline-rich 82 amino acid stretch, a C-terminal repeat segment comprised of ten 20-amino acid repeat sequences, and a 17-amino acid tail. A panel of MAbs to Rx1 PspA have been produced and the binding sites of nine of these Mabs were recently localized within the Rx1 *pspA* sequence in the alpha-helical region. Five of the Rx1 Mabs were protective in mice infected with a virulent pneumococcal strain, WU2. Four of these five protective antibodies were mapped to the distal third (amino acids 192-260) of the alpha-helical domain of Rx1 PspA.

Truncated PspAs containing amino acids 192-588 or 192-299, from pneumococcal strain Rx1 were cloned and the recombinant proteins expressed and evaluated for their ability to elicit protection against subsequent challenge with *S. pneumoniae* WU2. As with full-length Rx1 PspA, both truncated PspAs containing the distal alpha-helical region protected mice against fatal WU2 pneumococcal infection. However, the recombinant PspA fragment extending from amino acid 192 to 588 was more immunogenic than the smaller fragment, probably due to its larger size. In addition, the protection elicited by the amino acid fragment 192-588 of Rx1 was comparable to that elicited by full-length Rx1 PspA. Therefore, cross-protective epitopes of other PspAs were also sought in the C-terminal two-thirds of the molecule. As discussed below, PspAs homologous to amino acids 192-588 of strain Rx1 were amplified by PCR, cloned, and expressed in *E. coli*. Then three recombinant PspAs, from capsule type 4 and 5 strains, were evaluated for their ability to confer cross-protection against challenge strains of variant capsular types. The data demonstrate that the truncated PspAs from capsular type 4 and 5 strains collectively protect against or early death caused by challenge with capsular type 4 and 5 parental strains as well as type 3, 6A, and 6B *S. pneumoniae*.

<u>Bacterial strains and culture conditions</u>. All pneumococci were from the culture collection of this laboratory, and have been described (Yother, J. et al., Infect. Immun. 1982; <u>36</u>: 184-188; Briles, D.E., et al., Infect. Immun. 1992; <u>60</u>: 111-116; McDaniel, L.S., et al., Microb. Pathog. 1992; <u>13</u>: 261-269; and McDaniel, L.S, et al., In: Ferretti, J.J. et al., eds. <u>Genetics of streptococci, enterococci, and lactococci</u>. 1995; 283-286), with the exception of clinical isolates TJ0893, 0922134 and BG8740. Pneumococcal strains TJO893 and 0922134 were recovered from the blood of a 43-year old male and an elderly female, respectively. *S. pneumoniae* BG8743 is a blood isolate from an 8-month old infant. Strains employed in this study included capsular type 3 (A66.3, EF10197, WU2), type 4 (BG9739, EF3296, EF5668, L81905), type 5 (DBL5), type 6A (DBL6A, EF6796), type 6B (BG7322, BG9163, DBL1), type 14 TJO893), type 19 (BG8090), and type 23 (0922134, BG8743). In addition, strain WG44.1, which expresses no detectable PspA, was

employed in PspA-specific antibody analysis. All chemicals were purchased from Fisher Scientific, Fair Lawn, New Jersey unless indicate otherwise.

S. pneumoniae were grown in Todd Hewitt broth (Difco, Detroit, Michigan) supplemented with 5% yeast extract (Difco). Mid-exponential phase cultures were used for seeding inocula in Lactated Ringer's (Abbott laboratories, North Chicago, Illinois) for challenge studies. For pneumococcal strains used in challenge studies, inocula ranged from 2.8 to 3.8 $\log_{10}$ CFU (verified by dilution plating on blood agar). Plates were incubated overnight in a candle jar at 37°C.

E. coli DH1 and BL21(DE3) were cultured in LB medium (1% Bacto-tryptone (Difco), 0.5% Bacto Yeast (Difco), 0.5% NaCl, 0.1% dextrose). For the preparation of cell lysates, recombinant *E. coil* were grown in minimal E medium supplemented with 0.05 M thiamine, 0.2% glucose, 0.1% casamino acids (Difco), and 50 mg/ml kanamycin. Permanent bacterial stocks were stored at -80°C in growth medium containing 10% glycerol.

<u>Construction of plasmid-based strains</u>. pET-9a (Novagen, Madison, Wisconsin) was used for cloning truncated *pspA* genes from fourteen *S. pneumoniae* strains: DBL5, DBL6A, WU2, BG9739, EF5668, L81905, 0922134, BG8090, BG8743, BG9163, DBL1, EF3296, EF6796, and EF10197 (Table 1). *pspA* gene fragments, from fifteen strains, were amplified by PCR using two primers provided by Connaught Laboratories, Swiftwater, Pennsylvania Primer N192- 5'GGAAGGCCATATGCTCAAAGAGATTGATGAGTCT3' and primer C588 - 5'CCAAGGATCCTTAAACCCATTCACCATTGGC3' were engineered with *Nde*I and *Bam*HI restriction endonuclease sites, respectively. PCR-amplified gene products were digested with *Bam*HI and *Nde*I, and ligated to linearized pET-9a digested likewise and further treated with bacterial alkaline phosphatase United States Biochemical Corporation, Cleveland, Ohio) to prevent recircularization of the cut plasmid. Clones were first established

in *E. coli* BL21(DE3) which contained a chromosomal copy of the T7 RNA polymerase gene under the control of an inducible *lacUV5* promoter.

E. coli DH1 cells were transformed by the method of Hanahan (Hanahan, D. J. Mol. Biol. 1983; 166: 557-580). Stable transformants were identified by screening on LB-kanamycin plates. Plasmid constructs, isolated from each of these strains, were electroporated (Electro Cell Manipulator 600, BTX Electroporation System, San Diego, California) into *E. coli* BL21(DE3) and their respective strain designations are listed in Table 1. The pET-9a vector alone was introduced into *E. coli* BL21(DES) by electroporation to yield strain RCT125 (Table 2). All plasmid constructs and PCR-amplified *pspA* gene fragments were evaluated by agarose gel electrophoresis (with 1 kb DNA ladder, Gibco BRL, Gaithersburg, Maryland). Next, Southern analysis was performed using LM*pspA*1, a previously described full-length *pspA* probe (McDaniel. L.S. et al., Microb. Pathog. 1992; 13: 261-269) random primed labeled with digoxigenin-11-dUTP (Genius System, Boehringer Mannheim, Indianapolis, Indiana). Hybridization was detected with chemiluminescent sheets according to the manufacturer's instructions (Schleicher & Schuell, Keene, New Hampshire).

Cell fractionation of recombinant *E. coli* strains. Multiple cell fractions from transformed *E. coli* were evaluated for the expression of truncated PspA molecules. Single colonies were inoculated into 3 ml LB cultures containing kanamycin and grown overnight at 37°C. Next, an 80 ml LB culture, inoculated with 1:100 dilution of the overnight culture, was grown at 37°C to mid-exponential phase (A_{600} of ca. 0.5) and a 1 ml sample was harvested and resuspended (uninduced cells) prior to induction with isopropylthiogalactoside (IPTG, 0.3 mM final concentration). Following 1, 2, and 3 hr of induction, 0.5 ml of cells were centrifuges, resuspended, and labeled induced cells. The remaining culture was divided into two aliquots, centrifuged (4000 x g, 10 min, DuPont Sorvall RC 5B Plus), and the supernatant discarded. One pellet was resuspended in 5 ml of 20 mM Tris-HCl ph 7.4 200 mM NaCl, 1 mM (ethylenedinitrilo)-tetraacetic acid disodium salt (EDTA) and frozen at -20°C overnight. Cells were thawed at 65°C for 30 min, placed on ice, and sonicated for vive 10-sec pulses (0.4 relative output, Fisher Sonic Dismembrator, Dynatech Laboratories, Inc. Chantilly, Virginia). Next, the material was centrifuged (9000 x g, 20 min) and the supernatant was designed the crude extract-cytoplasmic fraction. The pellet was resuspended in Tris-NaCl-EDTA buffer and labeled the insoluble cell well and membrane fraction. The other pellet, from the divided induced culture, was resuspended in 10 ml of 30 mM Tris-HCl pH 8.0 containing 20% sucrose and 1 mM EDTA and incubated at room temperature for 10 min with agitation. Cells were then centrifuged, the supernatant removed, and the pellet resuspended in 5 mM $MgSO_4$ (10 ml, 10 min, shaking 4°C bath). This material was centrifuged and the supernatant was designated osmotic shock-periplasmic fraction. Cell fractions were evaluated by SDS-PAGE and immunoblot analysis.

MAbs to PspA. PspA-specific monoclonal antibodies (MAbs) XiR278 and 1A4 were used as previously described (Crain, M.J. et al., 1990, Infect. Immun.; 58: 3293-3299). MAb P50-92D9 was produced by immunization with DBL5 PspA. The PspA-specificity of MAb P50-92D9 was confirmed by Western Analysis by its reactivity with native PspAs from *S. pneumoniae* DBL5, BG9739, EF5668, and L81095 and its failure to recognize the PspA-control strain WG44.1.

SDS-PAGE and immunoblot analysis. *E. coli* cell fractions containing recombinant PspA proteins and biotinylated molecular weight markers (low range, Bio-Rad, Richmond, California) were separated by sodium dodecyl sulfate-polyacrylamide (10%; Bethesda Research Laboratories, Gaithersburg, Maryland) gel electrophoresis (SDS-PAGE) by the method of Laemmli (Laemmli, U.K. Nature 1970; 227: 680-685). Samples were first boiled for 5 min in sample buffer containing 60 mM Tris pH 6.8, 1% 2-B-mercaptoethanol (Sigma, St. Louis, Missouri), 1% SDS, 10% glycerol, and 0.01% bromophenol blue. Gels were subsequently transferred (1 hr, 100 volts) to nitrocellulose (0.45 mM pores, Millipore, Bedford, Massachusetts) as per the method of Towbin et al. Blots were blocked with 3% casein, 0.05% Tween 20 in 10 mM Tris, 0.1 M NaCl, pH 7.4 for 30 min prior to incubating with PspA-specific monoclonal antibodies diluted in PBST for 1 hr at 25°C. Next, the blot was washed 3 times with PBST before incubating with alkaline phosphatase-labeled goat anti-mouse immunoglobulin (Southern Biotechnology Associates, Inc., Birmingham, Alabama) for 1 hr at 25°C. Washes were performed as before and blots was developed with 0.5 mg/ml 5-bromo-4-chloro-3-indolyl phosphate and 0.01% nitro blue tetrazolium (Sigma) first dissolved in 150 μl of dimethyl sulfoxide and then diluted in 1.5 M Tris-HCl pH 8.8. Dot blots were analyzed similarly. Lysate samples (2 μl) were spotted on nitrocellulose filters (Millipore), allowed to dry, blocked, and detected as just described.

<u>Preparation of cell lysates containing recombinant PspA proteins</u>. Transformed *E. coli* strains RCT105, RCT113, RCT117, and RCT125 (Table 2) were grown in mid-exponential phase in minimal E medium before IPTG induction (2 mM final concentration, 2 hours, 37°C). Cultures were harvested by centrifugation (10 min at 9000 x g), resuspended in Tris-acetate pH 6.9, and frozen at -80°C overnight. Samples were thawed at 65°C for 30 min, cooled on ice, and sonicated. Next the samples were treated with 0.2 mM AEBSF (Calbiochem, La Jolla, California) at 37°C for 30 min and finally centrifuged to remove cell wall and membrane components. Dot blot

analysis was performed using PspA-specific MAbs to validate the presence of recombinant, truncated PspA molecules in the lysates prior to their use as immunogens in mice. Unused lysate material was stored at -20°C until subsequent immunizations were performed.

Mouse immunization and challenge. CBA/CAHN-XID/J mice (Jackson Laboratories, Bar Harbor, Maine), 6-12 weeks old, were employed for protection studies. These mice carry a X-linked immunodeficiency that prevents them from generating antibody to polysaccharide components, thus making them extremely susceptible to pneumococcal infection. Animals were immunized subcutaneously with cell lysates from *E coli* recombinant strains RCT105, RCT113, RCT117, and RCT125 (Table 2) in complete Freund's adjuvant for primary immunizations. Secondary injections were administered in incomplete adjuvant and subsequent boosts in dH_2O. Immunized and nonimmunized mice (groups of 2 to 5 animals) were challenged with *S. pneumoniae* strains A66.3, BG7322, DBL6A, WU2, DBL5, BG9739, and L81905 intravenously (tail vein) to induce pneumococcal sepsis. Infected animals were monitored for 21 days and mice that survived the 3-week evaluation period were designated protected against death and scored as surviving 22 days for statistical analysis. Protection that resulted in extension of life was calculated as a comparison between mean number of days to death for immunized versus pooled control mice (nonimmunized and RCT125 sham-immunized; total of 6-7 animals).

Determination of PspA serum levels. Mice were bled retro-orbitally following the secondary boost and again prior to challenge. Representative mouse titers were evaluated by enzyme-linked immunorsorbent assay (ELISA) using native, parental PspAs isolated from pneumococcal strains DBL5, BG9739, and L81905. PspAs were immobilized on microtiter plates by incubating in 0.5 $NaHCO_3$, 0.5 M Na_2CO_3pH9.5 at 4°C overnight. Alkaline phosphatase-labeled goat anti-mouse immunoglobulin (Southern Biotechnology Associates, Inc.) was used to detect mouse serum antibodies. Color development was with p-nitrophenyl phosphate (Sigma, 1 mg/ml) in 0.5 m $MgCL_2$ pH 9.8 with 10% diethanolamine and absorbance was read at 405 nm after a 30 min incubation. Reciprocal titers were calculated as the last dilution of antibody that registered an optical density value of 0.1. Sera from individual mice within a particular immunogen group were evaluated separately and then the respective titers from four mice per group were combined to obtain titer range (Table 3).

Statistics. The one-tailed Fisher exact and two sample rank tests were used to evaluate protection against death and extension of life in the mouse model.

Cloning of truncated *pspA* genes. Using primers N192 and C588, truncated *pspA* genes from fifteen diverse pneumococcal strains representing eight different capsular types (Table 1) were amplified by PCR. Even though variability exists in *pspA* genes from different strain, this result demonstrates that sufficient conservation exists between variant *pspA* genes to allow sequence amplification in all strains examined to date. Successful *pspA* PCR-amplification extended to all capsule types evaluated.

Fourteen of the amplified *pspA* genes were cloned and three clones containing truncated PspA molecules from pneumococcal strains DBL5, BG9739, and L81905 were further studies (Table 2). To verify the constructions, plasmids from recombinant *E. coli* strains (RCT105, RCT113, RCT117, and RCT125 (Table 2) were isolated, digested with *Nde*I and *BAMHI* restriction endonucleases, and electrophoresed in 1% agarose side-by-side with the PCR products used in their respective constructions (Figure 1A). The digestion reaction was complete for pRCT105, while pRCT113 and pRCT117 digestions were incomplete (lanes 5 and 7, respectively).

This gel was denatured and DNA transferred to nylon for Southern analysis. Figure 1B depicts the corresponding Southern blot probed with full-length Rx1*pspA* DNA. Lane 1 contains pRCT125, digested vector alone, which does not react with the pneumococcal DNA-specific probe, as expected. The *pspA*-specific probe hybridized with the PCT products and the digested plasmid inserts (see arrow, Figure 1B) as well as the partially undigested pRCT113 and pRCT117 (lane 5 and 7), confirming successful cloning of DBL5, BG9739, and L81905 *pspA* DNA. Constructions were similarly confirmed with the eleven additional recombinant strains containing truncated *pspA* genes from *S. pneumoniae* strains of different capsular and PspA types.

Expression of recombinant PspA in *E. coli* B121(De3). Transformed *E. coli* strains RCT105, RCT113, RCT117, and RCT125 were cultured to mid-exponential phase prior to the addition of IPTG to induce expression of the cloned, truncated *pspA* gene in each strain. A cell fractionation experiment was performed to identify the location of recombinant PspA proteins in transformed *E. coli* strains. Samples representing uninduced cells, included cells (1 hr, 2 hr, and 3 hr time intervals), the periplasmic fraction, the cytoplasmic fraction, and insoluble cell wall/membrane material were resolved by SDS-PAGE. Proteins were then transformed to nitrocellulose and Western analysis was performed using monoclonal antibodies specific for PspA epitopes.

Figure 2 reveals that both the cytoplasmic (lane 8) and the insoluble matter fractions (lane 9), from recombinant strain RCT 105, contain a protein of approximately 53.7 kDa that is recognized by MAb XiR278 that is not seen in the uninduced cell sample (lane 3). This protein increases in quantity in direct correlation with the length of IPTG induction (lanes 4-6; 1 hr, 2 hr, and 3 hr respectively). No truncated RCT105 PspA was found in the periplasmics fraction (lane 7), which was expected since the pET-9a vector lacks a signal sequence that would be necessary for directing proteins to the periplasm. The observed molecular weight (ca. 53.5 kDa) is larger than the predicted molecular weight for the 1.2 kb DBL5*pspA* gene product (43.6 kDa; Figure 1A, lane 4). Like full-length Rx1 PspA, the observed and predicted molecular weights for truncated PspAs do not agree precisely. In addition, immunoblot analysis was performed for recombinant *E. coli* strains RCT113, and RCT117 (using MAbs 1A4 and P50-92D, respectively) and similar results were obtained, while no cell fractions from control strain RCT125 were recognized by MAb XiR278.

Evaluating the protective capacity of recombinant, truncated PspAs. The truncated PspA proteins from strains RCT113, RCT117, and RCT105 were expressed and analyzed for their ability to generate cross-protection against a battery of seven *S. pneumoniae* strains. Control mice (non-immunized and RCT125 sham-immunized) and recombinant PspA-immunized mice were challenged with mouse-virulent strains A66.3, BG7322, DBL6A, WU2, DBL5, BG9739, and L81905. Table 3 presents the day of death for each infected mouse.

Immunization with truncated PspA from RCT113, RCT117, and RCT105 conferred protection against death for all mice challenged with capsular type 3 strains (A66.3 and WU2 (Table 3). The three truncated PspAs also provided significant protection against death with DBL6A, and BG7322 pneumococci (capsular types 6A and 6B, respectively). In addition, immunization with recombinant RCT113 PspA extended days to death in mice challenged with strains DBL5, BG9739, and L81905, while RCT117 PspA prolonged the lives of mice inoculated with BG9739 pneumococci (Table 3). Truncated BG9739 PspA elicited protection against all challenge strains (100%) evaluated in this study, while recombinant L81905 and DBL5 truncated PspAs conferred protection against death with 71% and 57% of *S. pneumoniae* challenge strains, respectively.

Anti-PspA antibody titers elicited by the three immunogens vary over approximately a 10-fold range (Table 3). The lowest antibody levels were elicited by RCT105 and this truncated PspA also elicited protection against the fewest number of challenge strains. RCT113 and RCT117 elicited three and nine time as much anti-PspA antibody, respectively. As expected, no antibody to PspA was detected in nonimmunized mice nor was specific-PspA antibody measured in mice immunized with the vector-only control strain (RCT125).

In summary, immunization with RCT113 and RCT117 PspAs protected mice against fatal challenge with capsular type 3 and 6A strains and extended life for mice inoculated with type 4, 5, and 6B pneumococci. RCT105 PspA immunization protected against fatal infection with capsular type 3 and 6B strains and prolonged time to death for type 6A *S. pneumoniae* but offered not protection against type 4 and 5 strains. These data demonstrate that truncated PspAs from capsular type 4 and 5 pneumococci collectively protect mice and ergo other hosts, such as humans, against or delay death caused by each of the seven challenge strains. In general, however, more complete protection was observed against strains of capsular type 3, 6A, and 6B than against type 4 and 5 *S. pneumoniae*.

PspA has been shown to be a protection-eliciting molecule of *S. pneumoniae*. Immunization with PspA has also been shown to be cross-protective, although eliciting more complete protection against certain strains than others. Thus, it is possible that a broadly protective PspA vaccine might need to contain PspAs of more than one pneumococcal strain. The distal third of the alpha-helical region of PspA has been identified as a major protective region of PspA. Moreover, this region is presented in a very antigenic form when expressed with the intact C-terminal half of the molecule. In this Example, the ability to use truncated PspA proteins homologous to the region of Rx1 PspA extending from amino acid residue 192 to the C-terminus at residue 588 is demonstrated.

The C-terminal two-thirds of PspA was cloned from fourteen strains by PCR amplification of a gene fragment of the appropriate size (1.2 kb) which hybridized with full-length Rx1 *pspA*. Successful PCR amplification extended to all capsule types analyzed. Thus, the C-terminal two-third of PspA may be amplified from many, if not all, pneumococcal capsule types with Rx1 *pspA*-specific primers. This technique is thus applicable to the development of antigenic immunological or vaccine compositions containing multiple PspA or fragments thereof.

Of these clones, three truncated PspA proteins were expressed and evaluated in mouse immunization studies to determine their ability to cross-protect against challenge with a variety of pneumococcal capsular types. All three recombinant PspAs elicited antibody reactive with their respective donor PspA and all three elicited protection against pneumococcal infection. Of the two truncated PspA proteins that elicited the highest antibody responses, 100% and 71% of the challenge strains were protected. RCT105 PspA, which elicited the lowest titers of PspA-specific antibody, yielded protection against 57% of *S. pneumoniae* strains evaluated. With all truncated PspAs, significant levels of protection were observed in four of the seven challenge strains. In fact, in all instances except for on (RCT105-immunized mice challenged with strain BG9739) the trend was for truncated PspA-immunization to elicit protection against pneumococcal challenge. These results demonstrate that truncated Rx1 PspA (amino acids 192-588) cross-protects mice against fatal *S. pneumoniae* WU2 challenge. More importantly, these data show that the homologous regions of diverse PspAs demonstrate comparable cross-protective abilities.

Strains of capsular type 4 and 5 were more difficult to protect against than were type 3, 6A and 6B pneumococcal strains. Serological differences in PspAs might affect cross-protection in some cases. Yet the difficulty in protecting against the type 4 and 5 strains used herein could not be explained on this basis, since the truncated PspA immunogens were cloned from the same three type 4 and 5 strains used for challenge. Both PspAs from the type 4 strains delayed death caused by one or both type 4 challenge strains but neither could prevent death caused by either type 4 pneumococcal strain. Moreover, the truncated PspA from the type 5 strain DBL5 elicited protection against death or delayed death with strains of capsular types 3, 6A and 6B but failed to protect against infection with its donor strain or either type 4 challenge strain.

There may be several reasons why the truncated PspAs from capsular type 4 and type 5 strains failed to protect against death even with their homologous donor *S. pneumoniae* strains. One possibility is that the type 4 and 5 strains chosen for study are especially virulent in the XID mouse model. XID mice fail to make antibodies to polysaccharides and are therefore extremely susceptible to pneumococcal infection with less than 100 CFU of most strains, including those of capsular type 3, 4, 5, 6A, and 6B. The increased mouse virulence of types 4 and 5 is apparent from the fact that in immunologically normal mice these strains have lower LD_{50}s and/or are more consistently fatal than strains of capsular types 3, 6A, or 6B.

Another possibility is that epitopes critical to protection-eliciting capacity with capsular type 4 and 5 strains are not present in the C-terminal two-thirds of PspA (amino acids 192-588), the truncated fragments used for immunization. The critical epitopes for these strains may be located in the N-terminal two thirds of the alpha-helical region of their PspA molecules. Finally, it is also possible that PspA may be less exposed on some *S. pneumoniae* strains than others. Strain Rx1 PspA amino acid sequence does not contain the cell wall attachment motif LPXTGX described by Schneewind et al. found in many gram-positive bacteria. Rather, PspA has a novel anchoring mechanism that is mediated by choline interactions between pneumococcal membrane-associated lipoteichoic acid and the repeat region in the C-terminus of the molecule. Electron micrographic examination has confirmed the localization of PspA on the pneumococcal surface and PspA-specific MAb data supports the accessibility of surface-exposed PspA. However, it is not known whether *S. pneumoniae* strains differ substantially in the degree to which different PspA regions are exposed to the surrounding environment. Nor is it known if the quantity of PspA expressed on the bacterial cell surface differs widely between strains.

Table 1. pspA recombinant strains categorized by pneumococcal capsular type.

Capsular Type	Parent Strains	Respective Recombinant Strains
3	WU2, EF10197	RCT111, RCT137
4	BG9739, EF5668	RCT113, RCT115
	L81905, EF3296	RCT117, RCT133
5	DBL5	RCT105
6A	DBL6A, EF6796	RCT109, RCT135
6B	BG9163, DBL1	RCT129, RCT131
14	TJO893*	none*
19	BG8090	RCT121
23	0922134, BG8743	RCT119, RCT123

*Truncated pspA amplified recently, not yet cloned

Table 2. Description of recombinant strains used in evaluating the protection-eliciting capacity of truncated PspAs in mice.

Recombinant Strain	Description	Capsule Type of Parent PspA
RCT 105	BL21(DE3) E.coli with pET-9a:DBL5	5
RCT 113	BL21(DE3) E.coli with pET-9a:BG9739	4
RCT 117	BL21(DE3) E.coli with pET-9a:L81905	4
RCT 125	BL21(DE3) E.coli with pET-9a (vector only)	

Table 3. Evaluation of the protection elicited by truncated <u>S. pneumoniae</u> PspA molecules in mice by days to death post-challenge*.

		Challenge Strain [capsular type] $\log_{10}$ dose in CFU)						
Immunizing recombinant PspA/PspA donor strain	Reciprocal anti-PspA titer+	A66.3 [type 3] (2.78)	WU2 [type 3] (3.57)	DBL6A [type 6A] (3.24)	BG7322 [type 6B] (3.11)	DBL5 [type 5] (3.81)	BG9739 [type 4] (3.56)	L81905 [type 4] (3.62)
RCT113/BG9739	5590-50,300	4x>21†	4x>21§	15,3x>21‡	12,13,16,>21‡	3,3,4,5†	5,5,5,7†	5,6,8,8†
RCT117/L81905	5590-150,900	4x>21†	4x>21§	7,16,2x>21†	10,12,13,>21†	3,3,4,4¶	4,5,13,>21†	3,4,6,8
RCT105/DBL5	1860-16,770	4x>21†	4x>21§	8,10,13,21‡	4x>21‡	2,2,2,>21	2,2,2,4	4,5,5,5
RCT125/ vector only	20-620	3,6,6,>21	2,3,3,>21	3,6,6,6	7,8,8,14	2,2,2,2	2,2,3,4,5	2,3,5,5
none	0	2,2,2	2,3	3,3,4	6,7,9	2,5	3,5	2,5

* Animals surviving the 3-week evaluation period were sacrificed and days to death recorded as >21 days. For statistical analysis, <u>P</u> values were calculated at 22 days for these fully protected mice.

\+ Range of four sera per group of mice; titers measured against native donor PspAs

† <u>P<</u>0.012

§ <u>P<</u>0.035

¶ <u>P<</u>0.057

Note: One-tailed Fisher exact and two sample rank tests were used for statistical analysis.

EXAMPLE 2 - LOCALIZATION OF PROTECTION-ELICITING EPITOPES AND PspA OF *S. PNEUMONIAE*

This Example, the ability of PspA epitopes on two PspA fragments (amino acids 192-588 and 192-299) to elicit cross-protection against a panel of diverse pneumococci is demonstrated. Also, this Example identifies regions homologous to amino acids 192-299 of Rx1 in 15 other diverse pneumococcal strains. The DNA encoding these regions was then amplified and cloned. The recombinant PspA fragments expressed were evaluated for their ability to elicit cross-protection against a panel of virulent pneumococci.

Bacterial strains and media conditions. *S. pneumoniae* strains were grown in Todd Hewitt broth with 0.5% yeast extract (THY) (both from Difco Laboratories, Detroit, Michigan) at 37°C or on blood agar plates containing 3% sheep blood at 37°C under reduced oxygen tension. *E. coli* strains were grown in Luria-Bertani medium or minimal E medium. Bacteria were stored at -80°C in growth medium supplemented with 10% glycerol. *E. coli* were transformed by the methods of Hanahan (Hanahan, D. J. Mol. Biol. 1983; 166: 557). Ampicillin (Ap) was used at a concentration of 100 μg/ml for *E. Coli*.

Construction of pIN-III-ompA3 and pMAL-based *E. Coli* strains. Recombinant plasmids pBC100 and pBAR416 that express and secrete *pspA* fragments from *E. Coli* were constructed with pIN-III-ompA3 as previously described (McDaniel, L.S. et al., Microb. Pathog. 1994; 17: 323).

The pMAL-p2 vector (New England Biolabs, Protein Fusion & Purification System, catalog #800) was used for cloning *pspA* gene fragments to amino acids 192-299 from strain Rx1 and from 7 other *S. pneumoniae* strains: R36A, D39, A66, BF9739, DBL5, DBL6A, and LM100. Amplification of the *pspA* gene fragments was done by the polymerase chain reaction (PCR) as described previously (McDaniel, L.S. et al., Microb. Pathog. 1994; 17: 323) using primers 5'CCGGATCCGCTCAAAGAGATTGATGAGTCTG3' [LSM4] and 5'CTGAGTCGACTGAGTTTCTGGAGCTGGAGC3' [LMS6] made with

*Bam*HI and *Sal*I restriction endonuclease sites, respectively. Primers were based on the sequence of Rx1 PspA. PCR products and the pMAL vector were digested with *BAMHI* and *Sal*I, and ligated together. Clones were transformed into *E. Coli* DH5α by the methods of Hanahan. Stable transformants were selected on LB plates containing 100μg/ml Ap. These clones were screened on LB plates containing 0.1 mM IPTG, 80 μg/ml X-gal and 100 μg/ml Ap and replica LB plates with 100 μg/ml Ap according to the manufacturer's instructions. The strain designations for these constructs are listed in Table 6. Positive clones were evaluated for the correct *pspA* gene fragment by agarose gel electrophoresis following plasmid isolation by the methods of Birnboim and Doly (Birnboim, H.C. et al., Nucl. Acids Res. 1979, 7: 1513). Southern analysis was done as previously described using a full-length *pspA* probe (McDaniel, L.S. et al., Microb. Pathog. 1994; 17: 323), randomly primed labeled with digoxigenin-11-dUTP (Genius System, Boehdinger Mannheim, Indianapolis, Indiana) and detected by chemiluminescence.

Expression of recombinant PspA protein fragments. For induction of expression of strains BC100 and BAR416, bacteria were grown to an optical density of approximately 0.6 at 660 nm at 37°C in minimal media, and IPTG was added to a final concentration of 2 mM. The cells were incubated for an additional 2 hours at 37°C, harvested, and the periplasmic contents released by osmotic shock. For strains BAR36A, BAR39, BAR66, BAR5668, BAR9739, BARL5, BAR6A and BAR100, bacteria were grown and induced as above except LB media + 10 mM. glucose was the culture medium. Proteins from these strains were purified over an amylose resin column according to the manufacturer's instructions (New England Biolabs, Protein Fusion & Purification System, Catalog #800). Briefly, amylose resin was poured into a 10 mL column and washed with column buffer. The diluted osmotic shock extract was loaded at a flow rate of approximately 1 mL/minute. The column was then washed again with column buffer and the fusion protein eluted off the column with column buffer containing 10 mM maltose. Lysates were stored at -20°C until further use.

Characterization of truncated PspA proteins used for immunization. The truncated PspA molecules, controls and molecular weight markers (Bio-Rad, Richmond, CA) were electrophoresed in a 10% sodium dodecyl (SDS) - polyacrylamide gel and electroblotted onto nitrocellulose. Rabbit polyclonal anti-PspA serum and rabbit antimaltose binding protein were used as the primary antibodies to probe the blots.

A direct binding ELISA procedure was used to quantitatively confirm reactivities observed by immunoblotting. For all protein extracts, osmotic shock preparations were diluted to a concentration of 3 μg/ml in phosphate buffered saline (PBS), and 100 μl was added to the wells of Immulon 4 microtitration plates (Dynatech Laboratories, Inc., Chantilly, VA). After blocking with 1.5% bovine serum albumin in PBS, unfractionated tissue culture supernates of individual MAbs were titered in duplicated by three-fold serial dilution through seven wells and developed using an alkaline phosphatase-labeled goat anti-mouse immunoglobulin secondary antibody (Southern Biotech Associates, Birmingham, AL) and alkalinephosphatase substrate (Sigma, St. Louis, MO). The plates were read at 405 nm in a Dynatech plate reader after 25 minutes, and the 30% end point was calculated for each antibody with each preparation.

Immunization and Protection Assays. Six to nine week old CBA/CAHN-XID/J (CBA/N) mice were obtained from the Jackson Laboratory, Bar Harbor, Maine. CBA/N mice carry an X-linked immunodeficiency trait, which renders them relatively unable to respond to polysaccharide antigens, but they do respond with normal levels of antibodies against protein antigens. Because of the absence of antibodies reactive with the phosphocholine determinant of C-polysaccharide in their serum, the mice are highly susceptible to pneumococcal infection. Mice immunized with the BC100 fragment were injected inguinally with antigen emulsified in CFA, giving an approximate dose of 3 μg of protein per mouse. Fourteen days later the mice were boosted intraperitoneally with 3 μg of antigen diluted in Ringer's lactate without adjuvant. Control mice were immunized following the same protocol with diluent and adjuvant, but no antigen. Mice immunized with the BAR416 fragment were injected with 0.2 ml at two sites in the sublinguinal area with antigen emulsified in CFA. The mice were boosted inguinally fourteen days later with antigen emulsified in IFA and were boosted a second time fourteen days later intraperioneally with 0.2 ml of antigen diluted in Ringer's lactate without adjuvant.

Mice that were immunized with the homologues of Rx1 BAR416 were immunized as described above. The control animals followed the same immunization protocol but received maltose binding protein (MBP) diluted 1:1 in CFA for their immunization and were also boosted with MBP.

Serum analysis. Mice were retro-orbitally bled with a 75 μl heparinized microhematocrit capillary tube (Fisher Scientific) before the first immunization and then once approximately 2 hours before challenge with virulent pneumococci. The serum was analyzed for the presence of antibodies to PspA by an enzyme-linked immunosorbent assay (ELISA) using native full-length R36A PspA as coating antigen as previously described (McDaniel, L.S. Microb. Pathog. 1994; 17: 323).

Intravenous infection of mice. Pneumococcal cultures were grown to late log phase in THY. Pneumococci were diluted to 10^4 CFU based on the optical density at 420 nm into lactated Ringer's solution. Seven days following the last boost injection for each group, diluted pneumococci were injected intravenously (tail vein) in a volume of 0.2 ml and plated on blood agar plates to confirm the numbers of CFU per milliliter. The final challenge dose was approximately 50-100 times the LD_{50} of each pneumococcal strain listed in Tables 4-6. The survival of the mice was followed for 21 days. Animals remaining alive after 21 days were considered to have survived the challenge.

Statistical analysis. Statistical significance of differences in days to death was calculated with the Wilcoxon two-sample rank test. Statistical significance of survival versus death was made using the Fisher exact test. In each case, groups of mice immunized with PspA containing preparations were compared to unimmunized controls, or controls immunized with preparations lacking PspA. One-tailed, rather than two-tailed, calculations were used since immunization with PspA or fragments of PspA has never been observed to cause a statistically significant decrease in resistance to infection.

Cloning into pMAL vector. Using primers based on the sequence of Rx1 PspA, LSM4 and LSM6, *pspA* gene fragments were amplified by PCR from fifteen out of fifteen pneumococcal strains examined. Seven of the eleven gene fragments were cloned into pMAL-p2 and transformed into *E. coli* (Table 6). The correct insert for each new clone was verified by agarose gel electrophoresis and Southern hybridization analysis. Plasmids from recombinant *E. coli* strains BAR36A, BAR39, BAR66, BAR9739, BARL5, BAR6A and BAR100 were isolated, digested with *Bam*HI and *Sal*I restriction endonucleases and electrophoresed on a 0.7% TBE agarose gel. The gel was then denatured and the DNA transferred to a nylon membrane for southern hybridization. The blot was probed with full-length Rx1 *pspA* DNA at high stringency conditions. The cloning of R36A, D39, A66, BG9739, DBL5, DBL6A and LM100 *pspA* DNA into pMal-p2 was confirmed by the recognition of all *Bam*HI and *Sal*I digested DNA inserts by the Rx1 probe.

Expression and conformation of truncated recombinant proteins. The transformed *E. coli* strains BAR36A, BAR39, BAR66, BAR9739, BARL5, BAR6A and BAR100 were grown in LB media supplemented with 10 mM glucose and induced with 2 mM IPTG for expression of the truncated PspA protein fused with maltose binding protein. Transformed *E. coli* strains BC100 and BAR416, which express PspA fragments fused to the OmpA leader sequence in the pIN-III-ompA3 vector, were grown in minimal medium and induced with 2 mM IPTG for expression. Both vectors, pIN-III-ompA3 and pMal-p2, are vectors in which fusion proteins are exported to the periplasmic space. Therefore, an osmotic shock extract from the pMal-p2 containing bacteria was then run over an amylose column for purification and resolved by SDS-PAGE western blotting. The western blot of the protein extracts from BAR36A, BAR39, BAR66, BAR9739, BARL5, BAR6A and BAR100 were recognized by a rabbit polyclonal antibody made to strain BC100 PspA. The apparent M_r of full-length PspA from WU2 is 91.5 kD. The M_r of maltose binding protein is 42 kD and the expected M^r for the PspA portion of the fusion is 12 kD. All extracts exhibited molecular weights that ranged from 54 to 80 kD. This range of molecular weights can be attributed to the variability of *pspA* among different pneumococcal strains. An ELISA, with plates coated with the various cloned fragments quantitatively confirmed the reactivities that were observed in the western blots with all protein extracts.

Protection and cross-protection against fatal pneumococcal infection elicited by cloned PspA fragments. CBA/N mice were immunized with the truncated PspA fragment encoded by pBC100, which is composed of amino acids 192 to 588 of Rx1 PspA, and challenged with 13 different *S. pneumoniae* strains representing 7 different capsular types (Table 4). With all 13 strains, the immunization resulted in protection from death or an extended time to death. With 10 of the strains the difference was statistically significant. With strains of capsular types 3, 6A, and 6B, all immunized mice were protected against death. Although there were fewer survivors in the case of capsular types 2, 4, and 5, the immunization with BC100 resulted in significant increases in times to death.

The BC100 immunization studies made it clear that epitopes C-terminal to residue 192 could elicit cross-protection. The BAR416 fragment, which includes amino acids 192-299, could elicit protection from fatal infection with a single challenge strain WU2. This Example shows the ability of BAR416 immunization to protect against the 6 strains that had been best protected against by immunization with BC100. Immunization with the BAR416 construct resulted in increased time to death for all 6 challenge strains examined (Table 5). BAR416 provided significant protection against death with WU2, A66, BG7322 and EF6796 pneumococci (capsular types 3, 3, 6B and 6A respectively). It also prolonged the lives of mice challenged with ATCC6303 and DBL6A pneumococci (capsular types 3 and 6A respectively). Serum from mice immunized with the BAR416 fragment yielded a geometric mean reciprocal anti-PspA ELISA titer to full-length Rx1 PspA of 750. Mice immunized with BC100 had geometric mean reciprocal titers of close to 2000, while non-immunized mice had anti-PspA titers of <10.

The above data indicates that the BAR416 fragment from Rx1 elicits adequate cross-reactive immunity to protect against diverse pneumococci and suggests that this region must be serologically conserved among PspAs. This hypothesis was confirmed by immunized with recombinant BAR416 homologous regions from the 7 different clones and then challenging with strain WU2 (Table 6). All 7 immunogens elicited significant protection. PspA fragments from capsular types 2 and 22 and the rough R36A strain elicited complete protection against death with all challenged mice. All of the other immunogens were able to extend the time to death of all the mice with the median days to death being 21 days or >21 days. Serum from mice immunized with the BAR416 homologous fragments had anti-PspA reciprocal titers that ranged from 260 to 75,800 with an average of 5700 while control animals immunized with only maltose binding protein had anti-PspA reciprocal titers of <10.

Antibody reactivities. All of the above immunization studies attest to the cross-reactivity of epitopes encoded by amino acids from position 192-299. This region includes the C-terminal third of the α-helical region and the first amino acids of the proline rich region. Other evidence that epitopes within this region are cross-reactive among different PspAs comes form the cross-reactivity of a panel of nine MAbs all of which were made by immunization with Rx1 PspA. The epitopes of four of the antibodies in the panel reacted with epitopes mapping between amino acids 192-260. The epitopes of the other five MAbs in the panel map between amino acids 1 and 115 (McDaniel, L.S., et al., Microb. Pathog. 1994; 17: 323). Each of these 9 MAbs were tested for its ability to react with 8 different PspAs in addition to Rx1. The 5 MAbs whose epitopes were located within the first 115 amino acids, reacted on average with only 1 other PspA. Three of the 5 in fact, did not react with any of the other 8 PspAs. In contrast the MAbs whose epitopes map between 192 and 260 amino acids each cross-reacted with an average of 4 of the 8 non-Rx1 PspAs, and all of them reacted with at least two non-Rx1 PspAs. Thus, based on this limited section of individual epitopes, it would appear that epitopes in the region from 192-260 amino acids are generally much more cross-reactive than epitopes in the region from 1-115 amino acids.

The BC100 fragment of Rx1 PspaA can elicit protection against the encapsulated type 3 strain WU2. Although the PspAs of the two strains can be distinguished serologically they are also cross-reactive (Crain, M.J., et al., Infect. Immun. 1990; 58: 3293). The earlier finding made it clear that epitopes cross-protective between Rx1 and WU2 PspAs exist. The importance of cross-reactions in the region C-terminal to residue 192 is demonstrated in this Example where 13 mouse virulent challenge strains have been used to elicit detectable protection against all of them. The first indication that epitopes C-terminal to position 192 might be able to elicit cross-protection came from our earlier study where we showed the MAbs Xi64, XiR278, XiR1323, and XiR1325, whose epitopes mapped between amino acids 192 and 260 of strain Rx1 PspA, could protect against infection with WU2. Moreover, immunization with PspA fragments from 192-588 and 192-299 were able to elicit protection against infection against WU2. This Example shows that the BC100 Rx1 fragment (192-588) elicits significant protection against each of 13 different mouse virulent pneumococci, thereby firmly establishing the ability of epitopes C-terminal to position 192 to elicit a protective response. The observation that a fusion protein containing amino acids 192-299 fused C-terminal to maltose binding protein could also elicit cross-protection, permits the conclusion that epitopes in this 107 amino acid region of PspA are sufficient to elicit significant cross-protection against a number of different strains.

Evidence that a comparable region of other PspAs is also able to elicit cross-protection cam from the studies where sequences homologous to the 192-299 region of Rx1 PspA were made from 5 other PspAs. All 5 of these fragments elicited significant protection against challenge with strain WU2. These data provide some suggestion for serologic differences in cross-protection elicited by the 192-299 region.

Based on present evidence, without wishing to be bound by any one particular theory, it is submitted that the PspAs in strains D39, Rx1 and R36A are identical. All of the 9 mice immunized with the 192-299 fragments from R36A and D39 survived challenge with WU2. Only LM100, one of the non-R36A/D39 PspAs, protected as high a percentage of mice from WU2. The difference in survival elicited by the R36A/D39 PspAs and the non-Rx1 related PspAs was statistically significant.

The data does indicate however, that all of the differences in protection against different strains are not due to differences in serologic cross-reactivity. BC100, which is made from Rx1, protected against death in 100% of the mice challenged with 7 different strains of *S. pneumonia*, but only delayed death with strain D39, which is thought to have the same PspA as strain Rx1. Thus, some of the differences in cross-protection are undoubtedly related to factors other than PspA cross-reactivity. Whether such factors are related to differences in virulence of the different strains in the hypersuceptible Xid mouse, or differences in requirements for epitopes N-terminal to amino acid 192, or difference in the role of PspA in different strains is not yet known.

These results suggest that a vaccine containing only the recombinant PspA fragments homologous with Rx1 amino acids 192-299 is effective against pneumococcal infection. Moreover, the results demonstrate that utility of PspA a.a. 192-299, a.a. 192-260 and DNA coding therefor, e.g. primers N192 or 588 (variants of LSM4 and LSM2) as useful for detecting the presence of pneumococciae by detecting presence of that which binds to the amino acid or to the DNA, or which is amplified by the DNA, e.g., by using that DNA as a hybridization probe, or as a PCR primer, or by using the amino acids in antibody-binding kits, assays or tests; and, the results demonstrate that a.a. 192-299 and a.a. 192-260 can be used to elicit antibodies for use in antibody-binding kits assays or tests.

Table 4 Protection of mice by immunization with BC100 from Rx1 PspA

			BC100 Immunogen			Controls			
Challenge Strain*	Capsule type	PspA type	# alive /# dead	% Survival	Median days alive	# alive /# dead	% Survival	Median days alive	P Value§
D39	2	25	0/5	0%	5	0/3	0%	2	0.02
WU2	3	1	4/0	100%	>21	0/3	0%	3	0.002
ATCC6303	3	7	5/0	100%	>21	0/5	0%	7	0.004
A66	3	13	4/0	100%	>21	0/3	0%	1	0.03
EF10197	3	18	5/0	100%	>21	0/3	0%	2	0.02
EF5668	4	12	1/3	25%	9	0/3	0%	4	N.S.
EF3296	4	20	1/3	25%	5	0/3	0%	3	N.S.
L81905	4	23	1/3	20%	4	0/6	0%	2	0.02
BG9739	4	26	0/4	0%	6.5	0/3	0%	2	N.S.
DBL5	5	33	0/5	0%	5	0/3	0%	2	0.02
BG7322	6	24	4/0	100%	>21	1/2	33.3%	6	0.03
EF6796	6A	1	4/0	100%	>21	0/3	0%	1	0.03
DBL6A	6A	19	5/0	100%	>21	0/3	0%	7	0.03

* Mice were challenged with approximately 10^3 CFU/mL of each strain

§P values were based on comparison of days alive by a one-tailed Wilcoxon 2 sample-rank test

Table 5 Protection of mice by immunization with BAR416 from Rx1 PspA

			BC100 Immunogen			Controls			
Challenge Strain[*]	Capsule type	PspA type	# alive /# dead	% Survival	Median days alive	# alive /# dead	% Survival	Median days alive	P Value[§]
WU2	3	1	4/1	80%	>21	0/3	0%	1	0.002
ATCC6303	3	7	2/3	40%	13	1/4	20%	4	0.048
A66	3	13	5/0	100%	>21	0/5	0%	2	0.004
BG7322	6	24	3/2	60%	>21	0/4	0%	7	0.02
EF6796	6A	1	3/2	60%	>21	0/5	0%	5	0.004
DBL6A	6A	19	0/5	0%	7	0/5	0%	2	0.008

Note, mice were challenged with about 10^3 CFU of each strain.

§P values were based on comparison of days alive by a one-tailed Wilcoxon 2 sample-rank test

Table 6 Protection of mice against *S. pneumoniae* WU2 by immunization with BAR416 Analogs of 7 PspAs

Immunogen	Parent Strain	Capsule type	PspA type	# alive / total#	% Survival	Median days alive	P value[*] vs. MBP
BAR36A	R36A	-	25	4/4	100%	>21	0.002
BAR39	D39	2	25	5/5	100%	>21	0.0008
BAR66	A66	3	13	7/8	88%	>21	<0.0001
BAR9739	BG9739	4	26	5/8	63%	>21	0.0002
BARL5	DBL5	5	33	4/8	50%	21	0.03
BAR6A	DBL6A	6A	19	3/8	60%	>21	0.05
BAR100	LM100	22	ND	5/5	100%	>21	0.0008
MBP	-	-	-	0/8	0%	2	-

[*] P values were based on comparison of days alove by one-tailed Wilcoxon 2 sample-rank test

Note, the PspA fragments used for immunization were cloned from products amplified with primers LSM4 and LSM6. In addition to the strains listed above, PCR reactions with LSM4 and LSM6 amplified products of the appropriate size strains BG9163, WU2, L81905, EF6796, EF5668, BG7376, BG7322 and BG5-8A.

Table 7 Reactivity of MAbs with PspAs of Different Pneumococci

Donor of test PspA			MAb mapping to 1-115 amino acids					MAb mapping to 192-260 amino acids			
Strain	Capsule	PspA	XI126	XIR1224	XIR1526	XIR35	XIR16	XIR1323	XI64	XIR278	XIR1325
	Type	Type	IgG2b	IgM	IgG2b	IgG2a	IgG2a	IgM	IgM	IgG1	IgG2a
Rx1	rough	25	++	++	++	++	++	++	++	++	++
ATCC101813	3	3	++	-	-	-	-	++	++	++	++
EF10197	3	18	-	-	-	-	-	-	-	++	+/-
BG9739	4	26	-	-	-	-	-	++	-	+	++
L81905	4	23	-	-	-	-	-	-	-	-	-
BG-5-8-A	6A	0	+/-	+	-	-	-	+	-	+	-
BG9163	6B	21	-	-	-	-	-	-	-	+	-
LM100	22	N.D.	+/-	-	-	-	-	-	-	-	-
WU2	3	1	++	-	-	-	-	++	++	++	++

Note, immunoblot analysis was carried out with the nine MAbs from this study from this study against a panel of nine different pneumococcal strains. Rx1 served as a positive control. The results are presented as ++ (strong reaction), + (weak, but clearly positive reation), +/- (difficult to detect), - (no reaction). The PspA of all strains gave a positive reaction with rabbit antiserum against PspA. N.D. means not determined. Mapping of epitopes was to fragments of strain Rx1 PspA.

EXAMPLE 3 - ISOLATION OF PspA AND TRUNCATED FORMS THEREOF AND IMMUNIZATION THEREBY

PspA is attached to the pneumococcal surface through a choline binding site on PspA. This allows for successful procedures for the isolation of FL-PspA. PspA can be released from the surface of pneumococci by elution with 2 percent choline chloride (CC), or by growth in a chemically defined medium (CDM) contained 1.2 percent CC (CDM-ET). Since CDM-ET superatants lack high concentrations of choline, the PspA released into them can be adsorbed to a choline (or choline analog) column and isolated be elution from the column with 2 percent choline chloride (CC).

This Example describes the ability to obtain PspA by these procedures, and the ability of PspA obtained by these procedures to elicit protection in mice against otherwise fatal pneumococcal sepsis. Native PspA from strains R36A, RX1, and WU2 was used because these strains have been used previously in studies of the ability of PspA to elicit protective immunity (see, e.g., Examples infra and supra). The first MAbs to PspA were made against PspA from strain R36A and the first cloned fragments of PspA and PspA mutants came from strain Rx1. Strain Rx1 was derived from strain R36A, which was in turn derived from the encapsulated type 2 strain, D39. PspAs from these three strains appears to be identical based on serologic and molecular weight analysis. Molecular studies have shown no differences in the *pspA* genes of strains D39, Rx1, and R36A. The third strain that provided PspA in this Example is the mouse virulent capsular type 3 strain WU2. Its PspA is highly cross-reactive with that from R36A and Rx1, and immunization with Rx1 and D39 PspA can protect against otherwise fatal infections with strain WU2.

S. pneumoniae

Strains of *S. pneumoniae* used in this study have been described previously (*Table* ^{8}). Bacteria were grown in either Todd-Hewitt broth with 0.5 percent yeast extract (THY), or a chemically defined medium (CDM) described previously [32], [43]. Serial passage of stock cultures was avoided. Strains were maintained frozen in THY + 20 percent glycerol and cultured from a scraping of the frozen culture.

Recovery of PspA from pneumococci

PspA is not found in the medium of growing pneumococci unless they have reached stationary phase and autolysis has commenced [36]. To release PspA from pneumococci three procedures were used. In one approach were grow pneumococci 100 ml of THY and collect the cells by centrifugation at mid-log phase. The pellet was washed three time in lactated Ringer's solution (Abbot Lab. North Chicago, IL), suspended in a small volume of 2 percent choline chloride in phosphase buffered saline (PBS) (pH 7.0), incubated for 10 minutes at room temperature, and centrifuged to remove the whole pneumococci. From immuboblots with anti Pspa MAb Xi126[48] at serial dilutions of the original culture, the suspended pellet, and the supernatant, it was evident that this procedure released about half of the PspA originally present on the pneumococci. Analysis of silver stained polyacrylamide gels showed this supernatant to contain proteins in addition to PspA[36].

The CDM used in the remaining two procedures was modified from that of Van der Rijn[43]. For normal growth it contained 0.03% CC. To cause PspA to be released during bacterial growth, the pneumococci were grown in CDM containing 1.2 percent choline chloride (CDM-CC), or in CDM containing 0.03 percent ethanolamine and only 0.000,001 percent choline (CDM-ET). In media lacking a normal concentration of choline the F-antigen and C-polysaccharide contain phosphoethanolamine rather than phosphocholine [49]. In CDM-CC and CDM-ET, PspA is released from the pneumococcal surface because of its inability to bind to the cholines in the lipoteichoic acids [36]. In addition to releasing PspA from the pneumococcal surface, growth in CDM-CC or CDM-ET facilitates PspA isolation by its other effects on the cell wall. In these media pneumococci do not autolyse[49], thus permitting them to be grown into stationary phase to maximize the yield of PspA. In these media septation does not occur and the pneumococci grown in long chains[36,49]. As the pneumococci reach stationary phase they die cease making Psp^A, and rapidly settle out. Preliminary studies, using serial dilution dot blots to quantitate PspA, indicated that the production of PspA ceases at about the time the pneumococci begin to settle out, with the formation of visible strands of the condensed pneumococcal chains. When the pneumococci began to settle out, the medium was recovered by centrifugation at 2900 x g for 20 minutes, and filtered with a low protein-binding filter (.45μ Nalgene Tissue Culture Filter #158-0045).

For growth in CDM-CC or CDM-ET, the pneumococci were first adapted to the defined medium and then, in the case of CDM-ET, to very low choline concentrations. To do this, strains were first inoculated into 1 part of THY and 9 parts of CDM medium containing 0.03 percent choline and 0.03 percent ethanolamine. After two subsequent subcultures in CDM containing 0.03 percent choline and 0.03 percent ethanoline (0.1 ml of culture + 0.9 ml of pre-warmed fresh medium), the culture was used to inoculate CDM with only 0.003 percent choline (and 0.03 percent ethanolamine). These steps was repeated until the strain would grow in CDM-ET containing 0.000,001 percent choline and 0.03 percent ethanolamine. It was critical that cultures be passed while in exponential growth phase (at about 10^7 CFU.ml). Even trace contamination of the medium by exogenous choline resulted in the failure of the PspA to be released from the pneumococcal surface[36]. Thus, disposable plastic ware was used for the preparation of CDM-ET media and for growth of cultures. Once a strain was adapted to CDM-ET it was froze in 80 percent CDM-ET and 20 percent glycerol at -80°C. When grown subsequently the strain was inoculated directly into the CDM-ET.

Isolation of native (full-length) PspA

PspA was isolated from the medium of cells grown in CDM-ET using choline-Sepharose prepared by conjugating choline to epoxy-activated Sepharose[50]. A separate column was used for media from different strains to avoid cross-contamination of their different PspAs. For isolation of PspA from clarified CDM-ET, we used a 0.6 ml bed volume of choline-Sepharose. The column bed was about 0.5 cm high and 1.4 cm in diameter. The flow rate during loading and washing was approximately 3 ml/min. After loading 300 ml CDM-ET supernatant, the column was washed 10 times with 3 ml volumes of 50 mM Tris acetate buffer, pH 6.9 containing 0.25 M NaCl (TAB). The washed column was eluted with sequential 3 ml volumes of 2 percent CC in TAB. Protein eluted from the column was measured (Bio-Rad protein assay, Bio-Rad, Hercules, CA). The column was monitored by quantitative dot blot. The loading material, washes, and the eluted material were dot blotted (1 μl) as undiluted, 1/4, 1/16, 1/64, 1/256, and 1/1024 on nitrocellulose. The membranes were then blocked with 1 percent BSA in PBS, incubated for 1 hr with PspA-specific MAbs Xi126 or XiR278, and developed with biotinylated goat-anti-mouse Ig, alkaline phosphatase conjugated streptavidin (Southern Biotechnology Associates Inc. Birmingham, AL), and nitrobluetetrazolium substrate with 5-bromo 4-chloro-3-indoyl phosphate *p*-toluidine salt (Fisher Scientific, Norcross GA)[17]. The purity of eluted PspA was assessed by silver-stained (silver stain kit, Bio Rad, Hercules, CA) SDS-PAGE gels run as described previously[32]. Immunoblots of SDS-PAGE gels were developed with MAbs Xi126 and XiR278[17].

Isolation of 29 kDa PspA

The 29 kDa fragment comprising the N-terminal 260 amino acids of PspA was produced in DH1 *E. coli* from pJY4306[31, 37]. An overnight culture of JY4306 was grown in 100 ml of Luria Broth (LB) containing 50μg/ml ampicillin. The culture was grown at 37°C in a shaker at 225 rpm. This culture was used to inoculate 6 one liter cultures that were grown under the same conditions. When the culture O.D. at 600 nm reached 0.7, 12 grams of cells, as a wet paste, were harvested at 4°C at 12,000 xg. The pellet was washed in 10 volumes of 25 mM Tris pH 7.7 at 0°C and suspended in 600 ml of 20% sucrose, 25 mM Tris pH 7.7 with 10 mM ethylenediamine tetraacetic acid (EDTA) for 10 minutes. The cells were pelleted by centrifugation (8000 xg) and rapidly suspended in 900 ml of 1 percent sucrose with 1 mM Pefabloc SC hydrochloride (Boehringer Mannheim Corp., Indianapolis, IN.) at 0° C. The suspension was pelleted at 8000 xg at 4°C. The precipitated from the periplasmic extract by 70 percent saturated ammonium sulfate overnight at 4°C for 30 minutes. The precipitated protein was resuspended in 35 ml of 20 mM histidine 1 percent sucrose at pH 6.6 (HSB). Insoluble materials were removed at 1,000 xg at 4°C for 10 minutes. The clarified material was dialyzed versus HSB, passed through a 0.2μm filter and further purified on a 1 ml MonoQ HR 5/5 column (Pharmacia Biotech, Inc., Piscataway, N.J.) equilibrated with HSB. The clarified material was loaded on the column at 1 ml/min, and the column was washed with 10 column volumes of HSB. The column was then eluted with a gradient change to 5 mM NaCl per minute at a flow rate of 1 ml/min. As detected by immuno blot with Xi126, SDS-PAGE and absorbance, PspA eluted as a single peak at approximately 0.27 to 0.30 M NaCl. By SDA-PAGE the material was approximately 90 percent pure. The yield from 6 liters of culture was 2 mg (Bio-Rad protein assay) of recombinant PspA.

Growth of pneumococci for challenge

Mice were challenged with log-phase pneumococci grown in THY. For challenge, the pneumococci were diluted directly into lactated Ringer's without prior washing or centrifugation. In inject the desired numbers of pneumococci, their concentration in lactated Ringer's solution was adjusted to an O.D. of about 0.2 at 420 nM (LKB Ultrospec III spectrophotometer). The number of pneumococci present was calculated at 5×10^8 CFU per ml/O.D. and confirmed by colony counts (on blood agar) of serial dilutions of the inoculum.

Immunization, challenge, and bleeding of mice

CBA/CAHN/XID/J (CB A/N) and BALB/cByJ (BALB/c) mice were purchased from Jackson Laboratory Bar Harbor, ME. Mice were given two injections two weeks apart and challenged i.v. two weeks later. Injections without CFA were given intrapertioneally in a 0.1 ml of Ringers. Where indicated, the first injection was given in complete Freund's adjuvant (CFA) consisting of approximately a 1:1 emulsion of antigen solution and CFA oil (Difco, Detroit MI). Antigen in CFA was injected inguinally in 0.2 ml divided between the two hind legs. All mice were boosted i/p. without adjuvant. When mice were injected with media supernatants or 2 percent choline chloride eluates of whole bacteria, the amounts of material injected were expressed as the volume of media from which the injected material was derived. For example, if the clarified medium from pneumococci grown in CDM-CC or CDM-ET was used for immunization without dilution or concentration, the dose was described as 100 μl. If the material was first diluted 1/10, or concentrated 10 fold, the dose was referred to as 10 or 1000 μl respectively.

ELISA for antibodies of PspA

Specific modifications of previously reported ELISA conditions[17], are described. Microtitration plates (Nunc Maxisorp, P.G.C. Scientific, Gaithersburg MD.) were coated with undiluted supernatants of Rx1 and WG44.1 pneumococci grown in CDM-ET or 1 percent BSA in PBS. Mice were bled retro-orbitally (75 μl) in a heparanized capillary tube (Fisher Scientific, Fair Lawn, N.J.) The blood was immediately diluted in 0.5 ml of one percent bovine serum albumin in PBS. The dilution of the resultant sera was 1/15 based on an average hematocrit of 47 percent. The sera were diluted in 7 three fold dilution in microtitration wells starting at 1/45. Mab Xi126 was used as a positive control. The maximum reproducible O.D. observed with Xi126 was defined as "maximum O.D." The O.D. observed in the absence of immune sera or MAb was defined as "minimum O.D." Antibody titers were defined as the dilution that gives 33 percent of maximum O.D. The binding to the Rx1 CDM-ET coated plates was shown to be PspA-specific, since in no case did we observe ≥33 percent of maximum binding of immune sera or Xi126 on plates coated with WG44.1 CDM-ET or BSA.

Statistical analysis

Unless otherwise indicated *P* values refer to comparisons using the Wilcoxin two-sample rank test to compare the numbers of days to death in different groups. Mice alive at 21 days were assigned a value of 22 for the sake of calculation. *P* values of >0.05 have been regarded as not significant. Since we have never observed immunization with PspA or other antigens to make pneumococci more susceptible to infection the *P* values have been calculated as single tailed tests. To determine what the *P* value would have been if a two tailed test had been used the values given should be multiplied by two. In some cases *P* values were given for comparisons of alive versus dead. These were always calculated using the Fisher exact test. All statistical calculations were carried out on a Macintosh computer using InStat(San Diego, CA). PspA is the major protection-eliciting component released from pneumococci grown in CDM-ET or CDM-CC, or released from conventionally grown pneumococci by elution with 2% CC.

PspA-containing preparations from pneumococci were able to protect mice from fatal sepsis following i.v. challenge with 3×10^3 ($100 \times LD_{50}$) capsular type 3 *S. pneumoniae (Table 9)*. Comparable preparations from the strains unable to elicit protection. Regardless of the method of isolation the minimum protective dose was derived from pneumococci grown in from 10-30 μl of medium. We also observed 9 that supernatants of log phase pneumococci grown in normal THY or CDM media could not elicit protection (data not shown). This finding is consistent with earlier studies[36, 37] indicating the PspA is not normally released in quantity into the medium of growing pneumococci.

Isolated PspA can elicit protection against fatal infection

Although PspA was necessary for these preparations to elicit protection it was possible that it did not act alone. Mice were thus, immunized with purified FL-PspA to address this question.

Isolation of FL-PspA from CDM-ET growth medium

We isolated the FL-PspA from CDM-ET rather than from CDM-CC medium or a 2 percent choline chloride elution of live cells, because the high levels of choline present in the latter solutions prevents adsorption of the PspA to the choline residues on the choline-Sepharose column. PspA for immunization was isolated from strain R36A, as the strain is non-encapsulated and the isolated PspA could not be contaminated with capsular polysaccharide. As a control we have conducted mock isolations from WG44.1 since this strain has an inactivated *pspA* gene and produces no PspA. The results shown in Table 10 are typical of isolations from 300 ml of CDM-ET medium from R36A grown pneumococci. We isolated 84 μg of PspA from 300 ml of medium, or about 280 μg/liter. Based on the dot blot results this appears to be about 75% of the PspA in the original medium, and that CDM-ET from R36A cultures contains about 400 μg/liter of PspA, or about 0.4 μg/ml.

No serologically detectable PspA was seen in the CDM-ET from WG44.1 cultures. More significantly there was undetectable protein recovered from the choline-Sepharose column after adsorption of CDM-ET from a WG44.1 culture, indicating that PspA is the only protein that could be isolated by this procedure. Moreover, by silver stained SDS PAGE gel the PspA isolated from R36A appeared to be homogenous *(Figure 3)*. Although autolysin can also be isolated on choline-Sepharose[20-50], we did not expect it to be isolated by this procedure since autolysin is not released from pneumococci grown in choline deficient medium[36]. The immunologic purity of the isolated PspA was emphasized by the fact that immunization with it did not elicit any antibodies detectable on plates coated with CDM-ET supernatants of WG44.1.

Loading more than 300 ml on the 0.6 ml bed volume column did not result in an increased yield, which suggested that the column capacity had been reached. However, increasing the depth of the choline-Sepharose bed to greater than 0.5 cm, decreased the amount of PspA eluted from the column, presumably because of non-specific trapping of aggregates in the column matrix. The elution buffer contains 50 mM Tris acetate 0.25 M NaCl and 2% choline chloride. Elution without added NaCl or with 1M NaCl resulted in lower yields. Elution with less than 1% CC also reduced yields.

Immunization o fmice with purified R36A PspA

For immunization we used only the first 3 ml fraction of the R36A column. Mice were immunized with two injections of 1, 0.1, or 0.01 μg o fR36A PspA, spaced two weeks apart. As controls, some mice were inoculated with comparable dilutions of the first 3 ml fraction from the WG44.1 column. Purified FL-PspA elicited antibody to

PspA at all doses regardless of whether CFA was used as an adjuvant (Table 11). In the absence of CFA the highest levels of antibody were seen with the 1 μg dose of PspA. In the presence of CFA, however, the 0.1 μg dose was as immunogenic as the 1 μg dose.

To test the ability of the different doses of the different doses of PspA to elicit protection against challenge we infected the immunized mice with two capsular type 3 strains, WU2 and A66. Although both of these strains are able to kill highly susceptible CBA/N XID mice at challenge doses of less than 10_2, the A66 strain is several logs more virulent when BALB/c mice are used [47,52]. The difference in virulence ofA66 and WU2, was partially compensated for by challenging the immunized CBA/N mice with lower doses of strain A66 than WU2.

After immunization of CBA/N mice with 1 and 0.1 μg doese of PspA we observed protection against WU2 challenge regardless of whether or not CFA was used as an adjuvant (Table 4). At the lowest dose, 0.01 μg PspA, most of the mice immunized with PspA + CFA lived whereas most immunized with PspA alone did not; however, the difference was not statistically significant. When immunized mice were challenged with the more virulent strain A66 [47,53], survivors were only observed among mice immunized with the 1 and 0.1 μg doses. There was slightly more protection against fatal A66 infection among mice immunized with CFA than without, but the difference was not statistically significant. When the two sample rank test was used to analyze the time to death of mice infected with A66 we observed a statistically significant delay in the time to death ineach immunized group as compared to the pooled controls.

The 29 kDa N-terminal fragment of PspA can elicit protection against infection when injected with CFA

We have compared the immunogenicity, with and without CFA, of an isolated 29 kDa fragment composed of the first 260 amino acids of PspA. Unlike the case with FL-PspA, adjuvant ws required for the 29 kDa fragment to elicit a protective response. This was observed even though the immunizing doses of the 29 kDa antigen used were 10 and 30 μg/mouse, or about 100 and 300 times the minimum does of FL-PspA that can elicits protection in the absence of adjuvant.

Injection with CFA revealed the presence of additional protection eliciting antigen(s) in CDM-CC, and CDM-ET growth medium but not in the 2 percent choline chloride eluates of live cells

The observation that Freud's adjuvant could have such a major effect on the immunogenicity of the 29 kDa fragment (Table 12), prompted us to reexamine the immunogens described in Table 2 to determine if immunization with adjuvant might enhance protection elicited by PspA-containing preparations or provide evidence for protection eliciting antigens in addition to PspA. By using CFA with the primary injection, the does of PspA-containing growth medium (CDM-CC and CDM-ET) required to elicit protection was reduced fro 10-30 μl (Table 9) down to 1 to 3 μl (Table 13). When CFA was used as an adjuvant with CDM-CC and CDM-ET from $PspA^-$ strains WG44.1 and JY1119 we were able to elicit protective immune responses if material from ≥100μl or more of media were injected. Thus, although there were apparently some protection eliciting components other than PspA in CDC-CC and CDM-ET growth media, PspA remained the major protection eliciting component even in the presence of adjuvant.

One of the media used for injection was CDM-ET in which JY2141 had been grown. This medium elicited protection against WU2 challenge even when injected at doses as low as 1 μl. It should be noted that although this strain does not make full-length PspA, it secretes a truncated molecule comprising the first 115 amino acids of PspA into the growth medium. Thus, unlike CDM-ET from

WG44.1 and JY1119, CDM-ET from JY2141 with 2 percent CC was relatively non-immunogenic even when emulsified with CFA. This result is consistent with the fact that the 115 amino acid N-terminal PspA fragment of JY2141 is not surface attached[37], and would be expected to be washed away prior to the elution with 2 percent CC.

Extension of studies to BALB/c mice i.p. challenge route

The studies above all involve i.v. challenge of CBA/N mice expressing with the XID genetic defect. The i.v. route, used in the present studies provides a relevant model for bacteremia and sepsis, but pneumococci have higher LD_{50s} when injected i.v. than i.p. CBA/N mice are hypersusceptible to pneumococcal infection because of the XIC defect. This genetic defect prevents them from having circulating naturally occurring antibody to phosphocholine. The absence of these antibodies have been shown to make XID mice several logs more susceptible to pneumococci than isogenic mice lacking the immune defect. From the data in Table 14 it is clear, however, that immunization with PspA can protect against infection in mice lacking the XID defect even when the challenge is by the i.p. route. Thus, there is no reason to suspect that the results presented are necessarily dependent on the use of the CBA/N XID mouse or the i.v. route.

PspA is highly immunogenic

These studies provide the first quantitative data on the amount of purified FL-PspA that is required to elicit protective immunity in mice. The isolated PspA for these studies was obtained by taking advantage of the fact that the C-terminal half of PspA binds to cell surface choline[36]. The isolated FL-PspA was found to be highly immunogenic in the mouse. Only two injections of 100 ng of PspA in the absence of adjuvant were reqired to elicit protection against otherwise fatal sepsis with greater than 100 Ld_{50} of capsulat type 3 *S. pneumoniae*. When the first injection was given with adjuvant, doses as small as 10 ng could elicit protective response The potent immunogenicity of PspA, and the ability to isolate it on choline-Sehparose columns provides a demonstration for the possible use of PspA as a vaccine in humans.

A large body of published [17, 29, 37] as well as unpublished evidence indicates that the major protection eliciting epitopes of PspA are located in the α-helical (N-terminal) half of the molecule. From the present studies, it is clear that immunization with N-terminal fragments containing the first 115 or 260 of the 288 amino acid α-helical region are able to elicit protection when given with CFA. However, these fragments were not able to elicit protective responses without CFA. In the case of the both the 115 and 260 amino acid fragments, even immunization at 100 times the minimum dose that is immunogenic for FL-PspA failed to elicit a protective response. This result is consistent with previous results showing that a fragment composed of the N-terminal 245 amino acids [31, 37] could elicit protection against otherwise fatal pneumococcal infection of mice when the immunization was given with CFA[32]. In that study no immunization without CFA was attempted. Even though the C-terminal half of PspA may not contain major protection-eliciting epitopes it appears to contain sequence important in the immunogenicity of the molecule as a whole, since the full length molecule elicited much greater protection than the N-terminal fragments. The effect of the C terminal half on antigenicity may be in part that it doubles the size of the immunogen. Molecules containing the C-terminal half of PspA may also be especially immunogenic because they exhibit more extensive aggregation than is seen with fragments expressing only the α-helical region [38]. Protein aggregates are known to generally be more antigenic and less tolerogenic than individual free molecules[54].

PspA is the major protection eliciting component of our pneumococcal extracts

Evidence that PspA is the major protection eliciting component of the CDM-ET, CDM-CC growth media and the two percent CC eluates was dependent on the use of mutant pneumococci that lacked the ability to produce FL-PspA. More than one pspA mutant strain was used to insure that the failure to elicit protection in the absence of FL-PspA was not a spurious result of non-PspA mutation blocking the production of some other antigen. Strains WG44.2 and JY1119 contain identical deletions that include the 5′ end of the pspA genes and extend about 3 kb upstream of pspA[37]. WG44.1 is a mutant of the non-encapsulated strain Rx1 and JY1119 was made by transforming capsular type 3 strain WU2 with the WG44.1 pspA mutation. In no case were preparations from WG4.1 and JY1119 as efficient at eliciting protection as those from the PspA+ strains. To rule out the possibility that protection elicited by preparations from the PspA+ strains was elicited by some non-PspA molecule also encoded by a 3kb deletion linked to the mutant pspA genes of WG44.1 and JY1119, we also used strains JY2141 and LM34[26,37]. In these strains the Rx1 pspA gene has been insertionally inactivated causing the production of N-terminal fragments of 115 and 245 amino acids respectively. These strains have no other known mutations. Although Rx1 and R36A are closely related non-encapsulated strains, some of the studies included Rx1 as the PspA+ control since it is the isogenic partner to WG44.1, LM34, and JY2141. The N terminal fragments produced by JY2141 and LM34 lack the surface anchor and are secreted into the medium [36]. Two percent CC eluates of JY2141 were non-protection eliciting even in the presence of adjuvant. In the absence of adjuvant, CDM-ET from JY2141 was not protection-eliciting. LM34 was tested without CFA in only 3 mice, but gave results consistent with those obtained with JY2141.

Anticapsular antibodies are known to be protective against pneumococcal infection [5,19]. However, in these studies it is unlikely they account for any of the protection we attributed to PspA. Our challenge strain bore the type 3 capsular polysaccharide and our primary source of PspA was strain R36A, which is a spontaneous non-encapsulated mutant of a capsular type 2 strain [39,41]. The 36A strain has been recently demonstrated to lack detectable type 3 capsule on the surface or in its cytoplasm [55]. Furthermore, the CBA/N mice used in most of the studies are unable to make antibody responses type 3 polysaccharide [56].

Non-PspA protection components

The observaiton that CDM-CC and CDM-ET supernatants of WG44.1 could elicit protection when injected in large amounts with adjuvant, suggested that these supernatants contained at least trace amounts of non-PspA protection eliciting molecules. In the case of preparations containing PspA eluted from the surface of live washed pneumococci with 2 percent CC, there was no evidence for any protection eliciting components other than PspA, presumably because the protection-eliciting non-PspA proteins released into the media were removed by the previous washing step. The identity of the protection eliciting molecules in the WG44.1 supernatant are unknown. In this regard, it is of interest that unlike R36A, strain Rx1 has been shown to contain a very small amount of cytoplasmic type 3 polysaccharide (but totally lacks surface type 3 polysaccharide [55]). This difference from Rx1 apparently came about through genetic manipulations in the contruction of Rx1 from R36A[39,41]. Thus, preparations made from Rx1 or from its daughter strains WG44.1, LM34, or JY2141, could potentially contain small amounts of capsular polysaccharide. For a number of reasons however, it seems very unlikely that the non-PspA protection-eliciting material identified in these studies was type 3 capsular polysaccharide (expressed by the WU2 challenge strain: 1) growth of these strains was either in CDM-CC or CDM-ET, each of which prevent autolysin activity and lysis [57] that would be required to release the small amount of type 3 polysaccharide from the cytoplasm of the Rx1 family strains; 2) CBA/N mice made protective responses to the non-PspA antigens, but express XID immune response deficiency which permits responses to proteins, but blocks antibody to most polysaccharides [46], including type 3 capsular polysaccharide [56]; and 3) immunogenicity of the non-PspA component required CFA, an adjuvant known to stimulate T-dependent (protein) rather than T-independent (polysaccharide) antibody responses.

A number of non-PspA protection eliciting pneumococcal proteins have been identified: pneumolysin, autolysin, neuraminidase, and PspA which are 52, 36.5, 107 and 37 kDa respectively [21,58,59,60]. The non-PspA protection eliciting components reported here could be composed of a mixture of these and/or other non-identified proteins. Attempts to identify lambda clones producing non-PspA protection eliciting proteins as efficacious as PspA have not been successful[25].

<u>Isolation of PspA</u>

The protection capacity CDM-CC, CDM-ET and material eluted from live cells with 2% CC were similar in terms of the volume of the original culture from which the injected dose was derived. The major advantage of eluting the PspA from the surface of pneumococci with 2 percent CC is that the pneumococci may be grown in any standard growth medium, and do not have to be first adapted to a defined medium. Moreover, concentration of PspA can be accomplished by centrifugation of th epneumococci prior to the elution of the PspA. An advantage of using either CDM-CC and CDM-ET media was that these media prevented lysis and pneumococci could be grown into stationary phase without contaminating the preparations with cytoplasmic contents and membrane and wall components. A particular advantage of CDM-ET growth medium is that since it lacks high concentrations of choline of PspA contained in it can be adsorbed directly to a choline-Sepharose column for affinity purification.

One liter of CDM-ET growth medium contains about 400 μg of PspA, and we were able to isolate about 3/4 of it to very high purity. At 0.1 μg/dose, a liter of CDM-ET contains enough PspA to immunize about 4,000 mice or possibly 40-400 humans. Our present batch size for a single column run is only 300 ml of CDM-ET. This could presumably be increased by increasing the amount of the adsorbent surface by increasing the diameter of the column. Using our present running buffer we have found that a choline-Sepharose resin depth of 0.5 cm was optimal; increases beyond 0.5 cm caused the overall yield to decrease rather than increase, even in the presence of larger loading of R36A CDM-ET.

Table 8 - Pneumococcal Strains

Strain	Capsule Type	PspA Expressed	Patent strain	Construction technique	References
D39	2	full length	-	clinical isolate	26,44
R36A	non-encapsulated	full length	D39	non-encapsulated mutuant	23,44,45
Rx1	non-encapsulated	full length	R36A	derived from R36A	26,39,41
WG44.1	non-encapsulated	none	Rx1	aberrant insertion activation with pKSD300	26,37
LM34	non-encapsulated	aa-1-245 of Rx1[a]	Rx1	insertional inactivation with pKSD300	26,37,42
JY2141	non-encapsulated	aa-1-115 of Rx1[a]	Rx1	insertional inactivation with pJY4208	37
WU2	3	full length	-	clinical isolate	25,46
JY119	3	none	WU2	transformation with WG44.1DNA	37
A66	3	full length	--	clinical isolate	44,47

[a] LM34 and LY2141 express fragments containing the first 245 and first 115 amino acids of Rx1 PspA respectively.

Table 9 - PspA is the major protection-eliciting component in antigen preparations made by three different methods

Preparation	Strain (PspA Status)	Dose as volume of media in μl[a]	Median Days Alive	Alive: Dead	P versus controls[b]
2% CC eluate from live cells	R36A (PspA$^+$)	1000	>21	2:0	
		200	>21	2:0	
		20	>21	2:0	
		2	1.5	0.2	
		all R36S	>21	6:2	0.03
	JY2141 (aa1-115)	1000	3,>21	1:1	
		200	1	0:2	
		20	1	0:2	
CDM-CC clarified medium	Rx1 (PspA$^+$)	100	>21	9:0	<0.0001
		30	>21	2:0	
		10	2	1:2	
		3	2	0:3	
		All	2,>21	12:6	0.0004
	LM34	100	2,2,>21	1:2	
	WG44.1 (pspA$^+$)	100	2	0:9	
		30	2	0:3	
		10	2	0:3	
		4	2	0:3	
	WU2 (pspA$^+$)	1000	>21	3:0	0.05
		100	>21	1:0	
		ALL	>21	4:0	0.03
	JY1119 (pspA$^+$)	1000	4	0:3	
	CDM-CC	100	2	0:2	
CDM-ET clarified medium	R36A (pspA$^+$)	100	>21	8:0	<0.0001
		10	3,>21	5:5	0.004
		1	1.5	3:5	
		0.1	2	0:2	
		ALL	>21	16:12	0.006
	JY2141 (aa1-115)	100	1.5	0:2	
		10	1.5	0:2	
	WG44.1 (pspA$^+$)	100	3	0:2	
		10	1.5	0:2	
None	-		2	0:14	-

[a] Antigen dose is given as the volume of growth media from which the 0.1 ml of injected material was derive. Each mouse was injected twice i.p. with the indicate doe diluted as necessary in lactated Ringer's injection solution.

[b] Controls used for statistical comparisons: 2% CC, all JY2141; CDM-CC Rx1, all WG44.1; CDM-CC WU2, JY2141 + all JY2141.

Table 10 - Isolation of PspA from 300ml of CDM-ET media after growth of R36A or WG44.1 pneumococci[a]

	R36A				WG44.1		
Fraction	μg protein/ml	total μg protein[b]	max reciprocal dot blot[c]	total dot blot units[b],[d]	μg protein per/ml	total μg protein[b]	max reciprocal dot blot[c]
growth media	13.3	3,990	4	1200	13.7	4,110	<1
fall-through	13.6	4,080	1	300	13.5	4,050	<1
1st wash			<1				<1
10th wash			<1				<1
elution #1	26	78	256	770	<1	-	<1
elution #2	2	6	16	48	<1	-	<1
elution #3	<1	-	4	12	<1	-	<1
total eluted		84		830		-	<1

[a] The columns were loaded with 300 ml of clarified CDM-ET medium after the growth of R36A or WG44.1. The column was washed with 10 sequential 3 ml fractions of TBA. Elution was with TBA plus 2 percent CC.

[b] Total μg protein or total dot blot units reflect the total in the 300 ml of the loading material or the 3 ml size of the eluted fractions.

[c] MAb XiR278 was used in the immunoblots to detect PspA in dot blots.

[d] Dot blot units were calculated as the reciprocal as the reciprocal dot titer times the volume in ml.

Table 11 - Purified full-length PspA is able to elicit protection against fatal sepsis in mice

			Anti-PspA	Challenge with $10^{5.1}$ WU2			Challenge with $10^{4.2}$ A.66		
Antigen	Dose[a]	Adjuvant or Diluent	titer[b] (Log mean ± S.E.)	Alive: Dead	Median Days Alive	P vs. pooled control[c]	Alive: Dead	Median Days Alive	P vs. pooled controls[c]
R36A (PspA$^+$)	1 μg	Ringer's	3.3 ± 0.2	5:0	>21	0.015	2:3	4	0.002
	0.1	Ringer's	2.6 ± 0.2	4:0	>21	0.041	1:4	4	0.0032
	0.01	Ringer's	2.7 ± 0.2	1:4	4	n.s.	0:5	3	0.0058
	1 μg	CFA	3.5 ± 0.2	5:0	>21	0.027	3:2	>21	0.0012
	0.1	CFA	3.6 ± 0.1	5:0	>21	0.013	2:3	4	0.0012
	0.01	CFA	3.1 ± 0.2	4:1	>21	0.015	0:5	3	0.0058
WG44.1 (PspA$^-$)	3600 μl	Ringer's	<1.6	n.d	n.d		1:4	3	n.s
	360	Ringer's	<1.6	n.d	n.d		0:5	2	n.s
	36	Ringer's	<1.6	n.d	n.d		0:5	2	n.s
	3600 μl	CFA	<1.6	n.d	n.d		0:5	2	n.s
	360	CFA	<1.6	n.d	n.d		1:4	2	n.s
	36	CFA	<1.6	n.d	n.d		0:5	2	n.s
saline	-	CFA	<1.6	1:5	4	---	n.d.	n.d.	-
pooled controls			<1.6	1:5	4		2:28	2	-

[a] For comparison with the data in Table 2, it should be noted that the 1, 0.1 and 0.01 μg doses were derived from 3600, 360, and 36 μl of R36A growth media. Equivalent dilutions of the PspA$^-$ eluate from strain WG44.1 were injected as controls. The amount of the WG44.1 preparations injected is listed as 3600, 360, and 36 μl and corresponds to the volume original growth medium from which the doses of WG44.1 was prepared.

[b] Antibody values were expressed as reciprocal ELISA titer.

[c] P values calculated by the Wilcoxon two sample rank test. By Kruskal-Wallis nonparametric ANOVA for the WU2 challenge was significant at P=0.01, for A66 significance was a P<0.0001.

Table 12. The 29 kDa N-terminal fragment of Rx1 PspA must be injected with adjuvant to elicit against WU2[a]

ug 29 kDa PspA	Adjuvant or diluent	Median Days Alive	Alive: Dead	*P* versus none[b]
30	CFA	>21	3 : 0	0.0006
3	CFA	>21	3 : 0	
30	Ringer's	2	0 : 3	
3	Ringer's	2	1 : 2	
none	CFA	2	0 : 7	
none	Ringer's	2	0 : 7	

[a] The 29 kDa fragment comprises the first 260 amino acids of PspA.

[b] For the calculation of *P* values the 30ug and 3 ug data were pooled; mice immunized with PspA + CFA were compared to CFA controls; mice immunized with PspA + Ringer's were compared to controls immunized with Ringers's. Only the statistically significant *P* values are shown. The calculated *P* value of PspA + CFA versus CFA alone, was 0.0006 by both the Wilcoxon two sample rank test and the Fisher exact test.

Table 13 PspA is not the only protection eliciting molecule released from pneumococci by interference with binding to choline on the surface of pneumococci

Preparation	Strain (PspA status)	Dose (as volume in ul)	Median Day Alive	Alive: Dead	P values[a]
					P vs. all JY2141
2% CC eulate from live cells	R36A (PspA$^+$)	1000	>21	2 : 0	0.02
		200	>21	5 : 0	0.02
		20	>21	5 : 0	0.02
		2	>21	5 : 0	0.001
		all R36A	>21	17 : 0	
	JY2141 (aa 1-115)	1000	>21	2 : 0	
		200	1	0 : 2	
		20	1	0 : 2	
		2	1	0 : 2	
		all JY2141	1	2 : 6	
					P versus pooled cont.
CDM-CC clarified medium + CFA	Rx1 (PspA$^+$)	1000	>21	3 : 0	0.002
		100	>21	3 : 0	0.002
	WU2 (PspA$^+$)	1000	>21	3 : 0	0.002
		100	>21	3 : 0	0.002
		3	>21	3 : 0	0.002
	WG44.1 (PspA$^-$)	1000	>21	5 : 1	<0.0001
		100	2.5	2 : 4	0.002
	JY1119 (PspA$^-$)	1000	>21	3 : 0	0.002
		100	>21	3 : 0	0.002
CDM-ET clarified medium + CFA	R36A (PspA$^+$)	1000	>21	3 : 1	0.004
		10	>21	4 : 0	0.004
		1	>21	3 : 1	0.004
		0.2	2	0 : 4	
	JY2141 (aa 1-115)	10	>21	2 : 0	
		1	>21	2 : 0	
	all JY2141	-	>21	4 : 0	0.004
	WG44.1 (PspA$^-$)	100	>21	2 : 0	
		10	2	0 : 2	
CDM-ET only	+ CFA		2	0 : 9	
None	none		1.5	0 : 4	
Pooled	Controls[b]		2	0 : 13	

[a] In cases where there were not statistically significant results no *P* value was shown.

[b] "Pooled Controls" refers to "CDM-ET only" Data and "None" data.

Table 14 Immunication of BALB/c mice with isolated PspA elicits protection against WU2 *S. Pneumoniae*

Antigen		Adjuvant or diluent	Challenge		Days to Death	P vs controls TSR/FE^b
Source	Dose^a		Log CFU	Route		
R36A (PspA$^+$)	1ug	CFA	4	i.p.	2,>21,>21,>21	0.06./0.03
WG44.1 (PspA$^-$)	100ul	CFA	4	i.p.	2,3	
None	-	CFA	4	i.p.	2,2,2,4	
R36A (PspA$^+$)	1ug	none	6	i.v.	2,>21,>21,>21	0.06./0.03
WG44.1 (PspA$^-$)	100ul	none	6	i.v.	5,7	
None	-	none	6	i.v.	2,2,2,3	
Pooled i.v and i.p. results					i.v. or i.p.	0.008/0.0007

[a] The 1ug dose of R36A PspA was isolated from 100 ul of CDM-ET medium. As a control mice were injected with an corresponding volume of choline-column effluent from a mock isolation of PspA from the PspA$^-$ strain WG44.1. The dose of WG44.1 material is expressed as 100 ul since this is the volume CDM-ET from which the injected column effluent was derived.

[b] *P* values calculated by Wilcoxon two-sample rank test, TSR, or Fisher exact, FE versus pooled controls for each groupd. "Pooled controls" include data obtained with by injection of "WG44.1" and "none". The i.p. and i.v. studies

EXAMPLE 4 - EVIDENCE FOR SIMULTANEOUS EXPRESSION OF TWO PspAs

From Southern blot analysis there has been an issue as to whether most isolates of *S. pneumoniae* has two DNA sequences that hybridize with both 5′ and 3′ halves of Rx1 pspA, or whether this is an artifact of Southern blot. When bacterial lysates have been examined by Western blot, the results have always been consistent with the production of a single PspA by each isolate. This Example provides evidence for the first time that two PspAs of different apparent molecular weights and different serotypes can be simultaneously expressed by the same isolate.

Different PspAs frequently share cross-reactive epitopes, and an immune serum to one PspA was able to recognize PspAs on all pneumococci. In spite of these similarities, PspAs of different strains can generally be distinguished by their molecular weights and by their reactivity with a panel of PspA-specific monoclonal antibodies (MAbs).

A serotyping system for PspA has been developed which uses a panel of seven MAbs. PspA serotypes are designated based on the pattern of positive or negative reactivity in immunoblots with this panel of MAbs. Among a panel of 57 independent isolates of 9 capsular groups/types, 31 PspA serotypes were observed. The large diversity of PspA was substantiated in a subsequent study of 51 capsular serotype 6B isolates from Alaska, provided by Alan Parkinson at the Arctic Investigations Laboratory of the Centers for Disease Control and Prevention. Among these 51 capsular type 6B isolates were observed 22 different PspAs based on PspA serotype and molecular weight variations of PspA.

While most pneumococcal strains appear to have two DNA sequences homologous with both the 5′ and 3′ halves of pspA, site-specific truncation mutations of Rx1 have revealed that one these, pspA, encodes PspA. The other sequence has been provisionally designated as the pspA-like sequence. At present whether the pspA-like sequence makes a gene product is unknown. Evidence that the pspA and pspA-like genes are homologous but distinct groups of alleles comes from Southern blot analysis at high stringencies. Additional evidence that pspA and the pspA-like loci are distinct comes from studies using PCR primers that permit amplification of a single product approximately 2Kb in size from 70% of pneumococci. For the remaining 30% of pneumococci no amplification was observed with the primers used.

Evidence for two PspAs:

When the strains of MC25-28 were examined with the panel of seven MAbs specific for different PspA epitopes, all four demonstrated the same patterns of reactivity (Fig. 4). The MAbs XiR278 and 2A4 detected a PspA molecule with an apparent molecular weight of 190 KDa in each isolate. In accordance with the previous PspA serotyping system, the 190 KDa molecule was designated as PspA type 6 because of its reactivity with XiR278 and 2A4, but none of the five other MAbs in the typing system. Each isolate also produced a second PspA molecule with an apparent molecular weight 82 KDa. The 82 KDs PspA in each isolate was detected only with the MAb 7D2 and was designated as type 34. No reactivity was detected with MAbs Xi126, Xi64, 1A4, or SR4W4. The fact that all four capsular 6B strains exhibit two PspAs, based on both molecular weights and PspA serotypes, suggested that they might be members of the same clone.

Simultaneous production of both PspAs:

Results from the colony immunobloting showed that both PspAs were present simultaneously in each colony of these isolates when grown *in vitro*. All colonies on each plate of the original culture, as well as all of the progeny colonies from a single colony, reacted with MAbs XiR278, 2A4, and 7D2.

Number of *pspA* genes:

One explanation for the second PspA molecule was that these strains contained an extra *pspA* gene. Since most strains contain a *pspA* gene and a *pspA*-like gene it was expected that if an extra gene were present one might observe at least three *pspA* homologous loci in isolates MC25-28. In *Hind* III digests of MC25-28 each strain revealed a 7.7 and 3.6 Kb band when probed with p1SM*pspA*13/2 (Figure 5A). In comparison, when Rx1 DNA was digested with *Hind* III and hybridized with p1SM*pspA*13.2, homologous sequences were detected on 9.1 and 4.2 Kb fragments as expected from previous studies (9) (Figure 5A). Results consistent with only two *pspA*-homologous genes in MC25-28 were also obtained with digestion using four additional enzymes (Table 15).

In previous studies it has been reported that probes for the 5′ half of *pspA* (encoding the alpha-helical half of the protein) bind the *pspA*-like sequence of most strains only at a stringency of around 90%. With chromosomal digests of MC25-28 we observed that the 5′ Rx1 probe of pLSM*pspA*12/6 bound both *pspA* homologous bands at a stringency of greater than 95 percent. The same probe bound only the *pspA* containing fragment Rx1 at a stringency above 95 percent (Figure 5B).

Further characterization of the *pspA* gene was done by RFLP analysis of PCR amplified *pspA* from each strain. Since previous studies indicated that individual strains yielded only one product, and since the amplification is carried out with primers based on a known *pspA* sequence, it seems likely that in each case the amplified products represent the *pspA* rather than the *pspA*-like gene. When MC25-28 were subjected to this procedure, an amplified *pspA* product of 2.1 Kb was produced in each case. When digested with *Hha* 1 digest the sum of the fragments obtained with each enzyme was approximately equal to the size of the 2.1 Kb amplified product (Figure 6). These results suggest that the 2.1 Kb amplified DNA represents the amplified product of only a single DNA sequence. Rx1, by comparison, produced an amplified product of 2.0 Kb and five fragments of 0.76, 0.468, 0390, 0.349 and 0.120, when digested with *Hha* 1 as expected from its known *pspA* sequence.

The four isolates examined in this Example are the first in which two PspAs have unambiguously been observed. The interpretation that two PspAs are simultaneously expressed by a single pneumococcal isolate is based on the observation that bands of different molecular weights were detected by different MAbs to PspA. Isolates used in this study were from a group originally selected for study by Brian Spratt because of their resistance to penicillin. It is very likely that all four of the isolates making two PspAs are related since they share PspA serotypes, amplified *pspA* RFLPs, chromosomal *pspA* RFLPs, capsule type, and resistance to penicillin.

The interpretation of studies presented here, showing the existence of two PspAs in the four strains MC25-28, must be set in the context of what is know about the serology PspA as detected by Western blots. PspAs of different strains have been shown previously to exhibit apparent molecular weight sizes ranging from 60 to 200 KDa as detected by Western blots. At least part of this difference in size is attributable to secondary structure. Even for the PspA of a single isolate, band of several sizes are generally observed. Mutation and immunochemistry studies have demonstrated, however, that all of the different sized PspA band from Rx1 are made by a single gene capable of encoding a 69 KDa protein. The heterogeneity of band size on Western blots of PspA made by a single strain appears to be due to both degradation and polymerization.

PspA was originally defined by reciprocal absorption studies demonstrating that a panel of MAbs to Rx1 surface proteins each reacted with some protein and later by studies using Rx1 and WU2 derivatives expressing various truncated forms of PspA. In both cases it was clear that each MAbs to the PspA of a given strain reacted with the same protein. Such detailed studies have not been done with each of the several hundred human isolates. It is possible that with some isolates, reactivity of the MAbs with two PspAs may have gone unnoticed. This could have happened if all reactive antibodies detected both PspAs of the same isolate, or if the most prominent migration bands from each of the two PspAs co-migrated. With isolates MC25-28 the observation of two PspAs was possible because clearly distinguishable bands of different molecular weights reacted preferentially with different MAbs.

Applicants favor the interpretation that isolates MC25-28 each make two PspAs, because an alternative possibility, namely, that the 190 KDa PspA detected by MAbs XiR278 and 2A4 might be a dimer of the 84 KDa monomer detected by MAb 7D2, if the epitopes recognized by the different MAbs were dependent on either the dimeric or monomeric status of the protein, seems unlikely since whenever MAbs react with the PspA of a strain, they usually detect both the monomeric and the dimeric forms. No other isolates have been observed where some MAbs detected only the apparent dimeric form of PspA while others detected only the monomeric form.

There could be several possible explanations for the failure to observe two PspAs produced by most strains. 1) All pneumococci might make two *pspAs* in culture, but MAbs generally recognize only one of them (perhaps in this isolate there has been a recombination between *pspa* DNA and the *pspA*-like locus, thus allowing that locus to make a product detected by MAb to PspA). 2) All pneumococci can have two *pspAs* but the expression of one of them generally does not occur under *in vitro* growth conditions. 3) The *pspA*-like locus is normally a nonfunctional pseudogene sequence that for an unexplained reason has become functional in these isolates.

It seems unlikely that the expression of only a single PspA by most strains is the result of a phase shift that permits the expression of only the *pspA* or *pspA*-like gene at any one time, since many of the strains examined repeatedly and consistently produce the same PspA. In the case of strains MC25-28, the appearance of two PspAs is apparently not the result of a phase switch, since individual colonies produced both the type 6 and the type 34 PspAs.

Presumably in these four strains, the second PspA protein is produced by the *pspA*-like DNA sequence. At high stringency, the probe comprising the coding region of the alpha-helical half of PspA recognized both *pspA* homologous sequences of MC25-28 but not the *pspA*-like sequence of Rx1. This finding indicates that the *pspA*-like sequence of MC25-28 is more similar to the Rx1 *pspA* sequence than is the Rx1 *pspA*-like sequence. If the *pspA*-like sequence of these strains is more similar to *pspA* than most *pspA*-like sequences, it could explain why we were able to see the products of *pspA*-like genes of these strains with our MAbs. The finding of two families of PspAs made *in vivo* by pneumococci, allows for use of the second PspA in compositions, as well as the use of DNA primers or probes for the second gene for more conclusive detecting, determining or isolating of pneumococci.

Isolates and Bacterial Cell Culture:

Pneumococcal isolates described in these studies were cultured from patients in Barcelona, Spain (one adult at Bellvitge Hospital, and three children at San Juan de Dios) between 1986 and 1988 (Table 2). These penicillin resistant pneumococci originally in the collection of Dr. Brian Spratt were shared with applicants by Dr. Alexander Tomasz at the Rockefeller Institute. Rx1 is a rough pneumococcus used in previous studies, and it is the first isolate in which *pspA* was sequenced. Bacteria were grown in Todd-Hewitt broth with

0.5% yeast extract or on blood agar plates overnight in a candle jar. Capsular serotype was confirmed by cell agglutination using Danish antisera (Statens Seruminstitut, Copenhagen, Denmark) as previously described. The isolates were subsequently typed as 6B by Quellung reaction, utilizing rabbit antisera against 6A or 6B capsule antigen prepared by Dr. Barry Gray.

Bacterial lysates:

Cell lysates were prepared by incubating the bacterial cell pellet with 0.1% sodium deoxycholate, 0.01% sodium dedecylsulfate (SDS), and 0.15 M sodium citrate, and then diluting the lysate in 0.5M Tris hydrochloride (pH 6.8) as previously described. Total pneumococcal protein in the lysates was quantitated by the bicinchonic acid method (BCA Protein Assay Reagent; Pierce Chemical Company, Rockford, IL).

PspA serotyping:

Serotyping of PspA was performed according to previously published methods. Briefly, pneumococcal cell lysates were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and developed as Western blots using a panel of seven MAbs to PspA. PspA serotypes were assigned based on the particular combination of MAbs with which each PspA was reactive.

Colony Immunoblotting:

A ten ml tube of Todd-Hewitt broth with 0.5% yeast extract was inoculated with overnight growth of MC23 from a blood agar plate. The isolate was allowed to grow to a concentration of 10^7 cells/ml as determined by an O.D. of 0.07 at 590nm. MC23 was serially diluted and spread-plated on blood agar plates to give approximately 100 cells per plate. The plates were allowed to grow overnight in a candle jar, and a single block agar plate with well-defined colonies was selected. Four nitrocellulose membranes were consecutively placed on the plate. Each membrane was lightly weighted and left in place for 5 minutes. In order to investigate the possibility of phase-variation between the two proteins detected on Western blots a single colony was picked from the plate, resuspended in ringers, and spread-plated onto a blood agar plate. The membranes were developed as Western blots according to PspA serotyping methods.

Chromosomal DNA Preparation:

Pneumococcal chromosomal DNA was prepared as in Example 9. The cells were harvested, washed, lysed, and digested with 0.5% (wt/vol) SDS and 100μg/ml proteinase K at 37°C for 1 hour. The cell wall debris, proteins, and polysccharides were complexed with 1% hexadecyl trimethyl ammonium bromide (CTAB) and 0.7M sodium chloride at 65°C for 20 minutes, then extracted with chloroform/isoamyl alcohol. DNA was precipitated with 0.6 volumes isopropanol, washed, and resuspended in 10mM Tris-HCL, 1mM EDTA, pH 8.0. DNA concentration was determined by spectrophotometric analysis at 260nm.

Probe preparation:

5′ and 3′ oligonucleotide primers homologous with nucleotides 1 to 26 and 1967 to 1990 of Rx1 *pspA* (LSM 13 and LSM2, respectively) were used to amplify the full length *pspA* and construct probe LSM*pspA*13/2 from Rx1 genomic DNA. 5′ and 3′ oligonucleotide primers homologous to nucleotides 161 to 187 and nucleotides 1093 to 1117 (LSM 12 and LSM 6, respectively) were used to amplify the variable alpha-helical region to construct probe LSM*pspA*12/6. PCR generated DNA was purified by Gene Clean (Bio101 Inc., Vista, CA) and random prime-labeled with digoxigenin-11-dUTP using the Genius 1 Nonradioactive DNA Labeling and Detection Kit as described by the manufacturer (Boehringer Mannheim, Indianapolis, IN).

DNA electrophoresis:

For Southern blot analysis, approximately 10μg of chromosomal DNA was digested to completion with a single restriction endonuclease, (*Hind* III, *Kpn* 1, *EcoR* 1, *Dra* 1, or *Pst* 1) then electrophoresed on a 0.7%

agarose gel for 16-18 hours at 35 volts. For PCR analysis, 5ul of product were incubated with a single restriction endonuclease, (*Bcl* 1, *Bam*H 1, *Pst* 1, *Sac* 1, *Eco*R 1 *Sma* 1, and *Kpn* 1) then electrophoresed on a 1.3% agrose gel for 2-3 hours at 90 volts. In both case, 1 Kb DNA ladder was used for molecular weight makers (BRL, Gaithersburg, MD) and gels were stained with ethidium bromide for 10 minutes and photographed with a ruler.

Southern blot hybridization

The DNA in the gel was depurinated in 0.25N HCL for 10 minutes, denatured in 0.5M NaOH and 1.5M NsCl for 30 minutes, and neutralized in 0.5M Tric-HCl (pH 7.2), 1.5M NaCl and 1mM disodium EDTA for 30 minutes. DNA was transferred to a nylon membrane (Micron Separations INC, MA) using a POSIBLOT pressure blotter (Strategene, La Jolla, CA) for 45 minutes and fixed by UV irradiation. The membranes were prehybridized for 3 hours at 42°C in 50% formamide, 5X SSC, 5X Denhardt solution, 25mM sodium phosphate (pH 6.5), 0.5% SDS 3% (wt/vol) dextran sulfate and 500μg/ml of denatured salmon containing 45% formamide, 5X SSC, 1X Denhardt solution, 20mM sodium phosphate (pH 6.5), 0.5% SDS, 3% dextran sulfate, 250μg/ml denatured sheared salmon sperm DNA and about 20ng of heat-denatured diogoxigenin-labeled probe DNA. After hybridization, the membranes were washed twice in 0.1% SDS and 2X SSC for 3 minutes at room temperature. The membranes were washed twice to a final stringency of 0.1% SDS in 0.3X SSC at 65°C for 15 minutes. This procedure yields a stringency greater than 95 percent. The membranes were developed using the Genius 1 Nonradioactive DNA Labeling and Detection Kit as described by the manufacturer (Boehringer Mannheim, Indianapolis, IN). To perform additional hybridization with other probes, the membranes were stripped in 0.2N NaOH/0.1%SDS at 40°C for 30 minutes and then washed twice in 2X SSC.

Polymerase Chain Reaction (PCR):

5′ and 3′ primers homologous with the DNA encoding the N- and C-terminal ends of PspA (LSM13 and LSM2, respectively) were used in these experiments. Amplifications were made using Taq DNA polymerase, $MgCl^2$ and 10X reaction buffer obtained from Promega (Madison, WI). DNA used for PCR was prepared using the method previously described in this paper. Reactions were conducted in 50ml volumes containing 0.2mM of each dNTP, and 1ml of each primer at a working concentration of 50mM. $MgCl_2$ was used at an optimal concentration of 1.75mM with 0.25 units of Taq DNA polymerase. Ten to thirty ng of genomic DNA was added to each reaction tube. The amplification reactions were performed in a thermal cycler (M.J. Research, Inc.) using the following three step program. Step 1 consisted of a denaturing temperature of 94°C for 2 minutes. Step 2 consisted of 9 complete cycles of a denaturing temperature of 94°C for 1 minute, an annealing temperature of 50°C for 2 minutes, and an extension temperature of 72°C for 3 minutes. Step 3 cycled for 19 times with a denaturing temperature of 94°C for 1 minute, an annealing temperature of 60°C for 2 minutes, and an extension temperature of 72°C for 3 minutes. At the end of the last cycle, the samples were held at 72°C for 5 minutes to ensure complete extension.

Band size estimation:

Fragment sizes in the molecular weight standard and in the Southern blot hybridization patterns were calculated from migration distances. The standard molecular sizes were fitted to a logarithmic regression model using Cricket Graph (Cricket Software, Malvern, PA). The molecular weights of the detected bands were estimated by entering the logarithmic line equation obtained by Cricket Graph into Microsoft Excel (Microsoft Corporation, Redmond, WA) in order to calculate molecular weights based in migration distances observed in the Southern blot.

TABLE 15

Restriction Enzyme	Strains Examined					Restriction Fragments (sizes in kilobases)	
	MC25	MC26	MC27	MC28	RX1	MC25-MC28	RX1
Hind III	+	+	+	+	+	7.7, 3.6	9.1, 4.2
Kpn I	+	+	+	+	+	11.6, 10.6	10.6, 9.8
EcoR I	+				+	8.4, 7.6	7.8, 6.6
Dra I	+				+	2.1, 1.1	1.9, 0.9
Pst I	+				+	>14, 6.1	10.0, 4.0

TABLE 16 Penicillin Resistant Capsular Serogroup 6 Strains from Spain

Isolate	Penicillin MIC (μg/ml)	Year	Site	Hospital
MC25	1	1986	sputum	Bellvitge
MC26	4	1988	ear	San Juan de Dios
MC27	1	1988	ear	San Juan de Dios
MC28	2	1988	?	San Juan de Dios

EXAMPLE 5 - SOUTHERN BLOT ANALYSIS OF *pspAs* AND FRAGMENTS OF *pspA*

In this example, Applicants used oligonucleotides derived from the DNA sequence of *pspA* of *S. pneumoniae* Rx1 both as hybridization probes and as primers in the polymerase chain reaction to investigate the genetic variation and conservation of the different regions of *pspA* and *pspA*-like sequences. The probes used ranged in size from 17 to 33 bases and included sequences representing the minus 35, the leader, the α-helical region, the proline-rich regions, the repeat regions, and the C-terminus. Applicants examined 18 different isolates representing 12 capsular and 9 PspA serotypes. The proline-rich, repeat, and leader, regions were highly conserved among *pspA* and *pspA*-like sequence.

In the previous Example, it was shown that strain Rx1 and most other strains of *S. pneumoniae* had two homologous sequences that could hybridize with probes encoding the N terminal and C terminal halves of PspA. This conclusion that these were separate sequences was supported by the fact that no matter which restriction enzymes was used there were always at least two (generally two sometimes three or four) restriction fragments of Rx1 and most other strains hybridized with the *pspA* probes. When the genome of Rx1 was digested with *Hind*III and hybridized with these, two *pspA*-homologous sequences were found to be in 4.0 and 9.1 kb fragments. Using derivative of Rx1 which had insertion mutations in *pspA*, it was possible to determine that the 4.0 kb fragment contained the functional *pspA* sequence. The *pspA*-homologous sequence included within the 9.1 kb band was referred to as the *pspA*-like sequence. Whether or not the *pspA*-like sequences makes a product is not know, and none has been identified *in vitro*. Since *pspA*-specific mutants can be difficult to produce in most strains, and exist for only a limited number of pneumococcal isolates, this Example identifies oligonucleotide probes that could distinguish between the *pspA* and *pspA*-like sequences.

The purpose of this Example was to further define both the conserved and variable regions of *pspA*, and to determine whether the central proline-rich region is variable or conserved, and identify those domains of *pspA* that are most highly conserved in the *pspA*-like sequence (and ergo, provide oligonucleotides that can distinguish between the two). Oligonucleotides were used and are therefore useful as both hybridization probes and as primers for polymerase chain reaction (PCR) analysis.

<u>Hybridization with oligonucleotide probes</u>

The oligonucleotides used in this study were based on the previously determined sequence of Rx1 PspA. Their position and orientation relative to the structural domains of Rx1 PspA are shown in Figure 7. The reactivity of these oligonucleotide probes with the *pspA* and *pspA*-like sequences was examined by hybridization with a *Hind*III digest of Rx1 genomic DNA (Table 17). As expected, each of the eight probes recognized the *pspA*-containing 4.0 kb fragment of the *Hind*III digested Rx1 DNA. Five of the 8 probes (LSM1, 2, 3, 7, and 12) could also recognize the *pspA*-like sequence of the 9.1 kb band at least at low stringency. At high stringency four of the probes (LSM2, 3, 4 and 5) were specific for the 4.0 kb.

These 8 probes were used to screen *Hind*III digest of the DAN from 18 strains of *S. pneumoniae* at low and high stringency. For comparison to earlier studies each of the strains was also screened using a full-length *pspA* probe. Table 23 illustrates the results obtained with each strain at high stringency. Table 18 summarizes the reactivities of the probes with the strains at high and low stringency. Strain Rx1 is a laboratory derivative of the clinical isolate, D39. The results obtained with both strains were identical. They are listed under a single heading in Table 23 and are counted as a single strain in Table 28. Although AC17 and AC94 are related clinical isolates, they have distinguishable *pspA*s and are listed separately. All of the other strains represent independent isolates.

The only strain not giving at least two *pspA*-homologous *Hind*III fragments was WU2. This observation was expected since WU2 was previously shown to have only one *pspA*-homologous sequence and to give only a single *Hind*III fragment that hybridizes with Rx1 *pspA*. Even at high stringency 6 of the 8 probes detected more than one fragment in at least one of the 18 strains Tables 18 and 23. Probes LSM7, 10 and 12 reacted with DNA from a majority of the strains and detected two fragments in over 59% of the strains they reacted with. In almost every case the fragments detected by the oligonucleotide probes were identical in size to those detected by the full-length *pspA* probe. Moreover, the same pairs of fragments were frequently detected by probes from the 3′ as well as the 5′ half Rx1 *pspA*. These results are consistent with earlier findings that the pairs of *Hind*III fragments from individual isolated generally include two separate but homologous sequences, rather than fragments of a single *pspA* gene.

The differences in the frequency with which the oligonucleotides reacted with (at least one fragment) of the strains in the panel was significant at $P < 0.0001$ by 2 x 8 chi square). When the oligonucleotides were compared in terms of their ability to react with both fragments of each strain the P value was also < 0.0001. Table 18 gives the percentage of strains reactive with each probe, the percentage in which only one fragment was reactive, and the percentage in which two (or more) fragments were reactive.

The last column in Table 18 give the ratio of strains that showed one reactive *Hind*III fragment at high stringency divided by the total number of reactive strains. In this column values of 1 were obtained with probes that only reacted with one band in each reactive strain. Such probes are assumed to be those that are most specific for *pspA*. The lowest values were obtained with probes that generally see two bands in each strain. Such probes are assumed to be those that represent regions relatively conserved between the *pspA* and *pspA*-like sequences. At high stringency, probes LSM3 and LSM4 detected only a single *Hind*III fragment in the DAN of strains they reacted with. These findings suggested probes LSM3 and LSM4 were generally detecting alleles of *pspA* rather than the *pspA*-like sequence. The observation that the fragments detected by LSM3 or LSM4 were also detected by all of the other reactive probes, strengthened the conclusion that these probes generally detected the *pspA* rather than the *pspA*-like sequence. WU2 has only one *pspA*-homologous DNA sequence and secretes a serologically detectable PspA. The fact that LSM3 reacts with the single *Hind*III fragment of WU2 is consistent with the interpretation that LSM3 detects the *pspA* sequences. Sequences representing the second proline region (LSM1) and the C-terminus (LSM2) appeared to also be relatively specific for the *pspA* sequences since they were generally detected in only one of the *Hind*III fragments of each strain.

Oligonucleotides, LSM12, and LSM10 detected the most conserved epitopes of *pspA* and generally reacted with both *pspA*-homologous fragments of each strain (Table 18). LSM7 was not quite as broadly cross-reactive but detected two PspAs in 41% of strains including almost 60% of the strains it reacted with. Thus, sequences representing the leader, first proline region, and the repeat region appear to be relatively conserved not only within *pspA* but between the *pspA* and *pspA*-like sequences. LSM3, 4, and 5 reacted with the DNA from the smallest fraction of strains of any oligonucleotide (29 - 35 percent), suggesting that the portion of *pspA* encoding the α-helical region is the least conserved region of *pspA*.

With two strains BG85C and L81905, the oligonucleotides detected more than two *Hind*III fragments containing *pspA*-homologous sequences. Because of the small size of the oligonucleotide probes and the absence of *Hind*III restriction sites within any of them, it is very unlikely that these multiple fragments were the results of fragmentation of the target DNA within the probed regions. In almost every case the extra oligonucleotides were detected at high stringency by more than one oligonucleotide. These data strongly suggest that at least in these two strains there are 3 or 4 sequences homologous to at least portions of the *pspA*. The probes most reactive with these additional sequences are those for the leader, the α-helical region and the proline rich region. The evidence for the existence of these additional *pspA*-related sequences was strengthened by results with BG58C and L81905 at low stringency where the LSM3 (α-helical) primer picked up the extra 1.2 kb band of L81905 (in addition to the 3.6 kb band) and the LSM7 (proline-rich) primer picked up the extra 3.2 and 1.4 kb bands (in addition to the 3.6 kb band) of BG58C.

Amplification of *pspA*

The utility of these oligonucleotides as PCR primers was examined by determining if they could amplify fragments of *pspA* from the genomic DNA of different pneumococcal isolates. Applicants attempted to amplify *pspA*s from 14 diverse strains of *S. pneumoniae* comprising 12 different capsular types using primers based on the Rx1 *pspA* sequence. Applicants observed that the 3′ primer LSM2, which is located at the 3′ end of *pspA*, would amplify an apparent *pspA* sequence from each of the 14 pneumococcal strains when used in combination with LSM1 located in the region of *pspA* encoding the proline-rich region (Table 19). LSM2 was also used in combination with four other 5′ primers LSM1, 3, 7, 8 and

12. LSM8 is located 5′ of the *pspA* start site (near the -35 region).

If a predominant sequence of the expected length was amplified that could be detected on a Southern blot with a full-length *pspA* probe, we assumed that *pspA* gene of the amplified DNA had homologous sequences similar to those of the *pspA* primers used. Based on these criteria the primer representing the α-helical sequence was found to be less conserved than the primers representing the leader, proline, and C-terminal sequences. These results were consistent with those observed for hybridization. The lowest frequency of amplification was observed with LSM8 which is from the Rx1 sequence 5′ of the *pspA* start site. This oligonucleotide was not used in the hybridization studies.

Further evidence for variability comes from differences in the sizes of the amplified *pspA* gene. The Example showed that when PCR primers LSM12 and LSM2 were used to amplify the entire coding region of PspA, PCR products from different pneumococcal isolates ranged in size from 1.9 and 2.3 kb (Table 20). The regions within *pspA* encoding the α-helical, proline-rich, and repeats were also amplified from the same isolates. As seen in Table 20, the variation in size of *pspA* appeared to come largely from variation in the size of *pspA* encoding encodes the α-helical region.

Using probes that consisted of approximately the 5′ and 3′ halves of *pspA* it has been determined that the portion of *pspA* that encodes the α-helical regions is less conserved than the portion of *pspA* that encodes the C-terminal half of the molecule. This Example show using 4 oligonucleotide probes from within each half of the DNA encoding PspA. Since a larger number of smaller probes were used, Applicants have been able to obtain a higher resolution picture of conserved and variable sequences within *pspA* and have also been able to identify regions of likely differences and similarities between *pspA* and the *pspA*-like sequences.

The only strains in which the *pspA* gene has been identified by molecular mutations are Rx1, D39 and WU2. Rx1 and D39 apparently have identical *pspA* molecules that are the result of the common laboratory origin of these two strains. WU2 lacks the *pspA*-like gene. Thus, when most pneumococci are examined by Southern blotting using full length-*pspA* as a probe, it is not possible to distinguish between the *pspA* and *pspA*-like loco, since both are readily detected. A major aim of these studies was to attempt to identify conserved and variable regions within the *pspA* and *pspA*-like loci. A related aim was to determine whether probes based on the Rx1 *pspA* could be identified that would permit one to differentiate *pspA* from the *pspA*-like sequence. Ideally such probes would be based on relatively conserved portion of the *pspA* sequence that was quite different in the *pspA*-like sequence. A useful *pspA* specific probe would be expected to identify the known Rx1 and WU2 *pspA* genes and identify only a single *Hind*III fragment in most other strains. Two probes (LSM3 and LSM4) never reacted with more than one *pspA*-homologous sequence in any particular strain. Both of reacted with Rx1 *pspA* and LSM3 reacted with WU2 *pspA*. Each of these probes reacted with 4 of the other 15 strains. When these probes identified a band, however, the band was generally also detected by all other Rx1 probes reactive with that strain's DNA. Additional evidence that the LSM3 and LSM4 were restricted to reactivity with *pspA* was that they reacted with the same bands in all three non-Rx1 strains. Each probe identifies *pspA* in certain strains and even when used in combination they recognized *pspA* in over 40 percent of strains. Probes for the second proline-rich region (LSM1) and the C-terminus of *pspA* (LSM2) generally, but not always, identified only one *pspA*-homologous sequence at high stringency. Collectively

LSM1, 2, 3, and 4 reacted with 16 of the 17 isolates and in each case revealed a consensus band recognized by most to all of the reactive probes.

By making the assumption that in different strains the Rx1 *pspA* probes are more likely to recognize *pspA* than the *pspA*-like sequences, it is possible to make some predictions about areas of conservation and variability within the *pspA* and *pspA*-like sequences. When a probe detected only a single *pspA*-homologous sequence in an isolate, it was assumed that it was *pspA*. If the probe detected two *pspA*-homologous sequences, it was assumed that it was reacting with both the *pspA* and *pspA*-like sequence. Thus, the approximate frequency with which a probe detects *pspA* can be read from Table 18 as the percent of strains where it detects at least one *pspA*-homologous band. The approximate frequency with which the probes detect the *pspA*-like sequence is the percent of strains in which two or more *pspA*-homologous band are detected.

Using these assumptions the most variable portion of portion of the *pspA* gene was observed to be the -35 region and the portion encoding α-helical region. The most conserved portion of *pspA* was found to be the repeat region, the leader and the proline rich region. Although only one probe from the region was used, the high degree of conservation among the 10 repeats in the Rx1 sequence makes it likely that other probes for the repeat regions give similar results.

The portion of the *pspA*-like sequence most similar to Rx1 *pspA* was that encoding the leader sequence, the 5′ portion of the proline rich region, and the repeat region, and those portions encoding the N-terminal end of the proline-rich and repeat regions. The repeat region of PspA has been shown to be involved in the attachment to PspA to the pneumococcal surface. The conservation of the repeat region among both *pspA* and *pspA*-like genes suggests that if is PspA-like protein is produced, that it may have a surface attachment mechanism similar to that of PspA. The need for a functional attachment site may explain the conservation of the repeat region. Moreover, the conservation in DNA encoding the repeat regions of the *pspA* and *pspA*-like genes suggests that the repeat regions may serve as a potential anti-pneumococcal drug target. The conservation in the leader sequence between *pspA* and the *pspA*-like sequence was also not surprising since similar conservation has been reported for the leader sequence of other gram positive proteins, such as M protein of group A streptococci. It is noteworthily, however, that there is little evidence at the DNA level that the PspA lead is shared by many genes other than PspA and the possible gene product of the *pspA*-like locus.

Although the region encoding the C-terminus of *pspA* (LSM12) or the 3′ portion of the proline-rich sequence (LSM1) appear to be highly conserved within *pspA* genes, corresponding regions in the *pspA*-like sequences are either lacking, or very distinct from those in *pspA*. The reason for conservation at these sites is not apparent. In the case of the PspA, its C-terminus does not appear to be necessary for attachment, since mutants lacking the C-terminal 49 amino acids are apparently as tightly attached to the cell surface as those with the complete sequence. Whether these difference from *pspA* portends a subtle difference in the mechanism of attachment of proteins produced by these two sequences in unknown. If the C-terminal end of the *pspA*-like sequence, or the 3′ portion of the proline-rich sequence in the *pspA*-like sequence are as conserved within the *pspA*-like family of genes as it is within *pspA*, then this region of *pspA* and the *pspA*-like sequence serve as targets for the development of probes to distinguish between all *pspA* and *pspA*-like genes.

With two strains, some of the oligonucleotide probes identified more than two *pspA*-homologous sequences. In the case of each of these strains, there was a predominant sequence recognized by almost all of the probes, and two or three additional sequences that were each recognized by at least two of the probes. One interpretation of the data is that there may be more than two *pspA*-homologous genes in some strains. The significance of such sequences is far from established. It is of interest however, that although the additional sequences is far from established. It is of interest however, that although the additional sequences share areas of homology with the leader, α-helical, and proline region, they exhibited no homology with the repeat region of the C-terminus of *pspA*. These sequences, thus, might serve as elements that can recombine with *pspA* and/or the *pspA*-like sequences to generate sequence diversity. Alternatively the sequences might produce molecules with very different C-terminal regions, and might not be surface attached. If these *pspA*-like sequences make products, however, they, like PspA, may be valuable as a component of a pneumococcal antigenic, immunological vaccine compositions.

Bacterial strains, growth conditions and isolation of chromosomal DNA

S. pneumoniae strains used in this study are listed in Table 5. Strains were grown in 100 ml of Todd-Hewitt broth with 0.5% yeast extract at 37°C to an approximate density of 5×10^8 cells/ml. Following harvesting of the cells by centrifugation (2900xg, 10 minutes), the DNA was isolated as previously described and stored at 4°C in TE (10mM Tris, 1mM EDTA, pH 8.0).

Amplification of *pspA* sequences

Polymerase chain reaction (PCR) primers, which were also used as oligonucleotide probes in Southern hybridizations, were designed based on the sequence of *pspA* from pneumococcal strain Rx1. These oligonucleotides were obtained from Oligos Etc. (Wilsonville, OR) and are listed in Table 22.

PCRs were done with a MJ Research, Inc., Programmable Thermal Cycler (Watertown, MA) as previously described using approximately 10 ng of genomic pneumococcal DAN with appropriate 5′ and 3′ primer pair. The sample was brought to a total volume of 50 µl containing a final concentration of 50mM KCl, 10mM Tris-HCl (PH 8.3), 1.5 mM $MgCl_2$, 0.001% gelatin, 0.5 mM each primer, 200mM of each deoxynucleotide triphosphate, and 2.5 U of *Taq* DNA polymerase. Following overlaying of the samples with 50 µl of mineral oil, the samples were denatured at 94°C for 2 minutes. Then the samples were subjected to 10 cycles consisting of 1 minute at 94°C, 2 minutes at 50°C, and 3 minutes at 72°C followed by another 20 cycles of 1 minute at 94°C, 2 minutes at 50°C, and 3 minutes at 72°C followed by another 20 cycles of 1 minute at 94°C, 2 minutes at 60°C, and 3 minutes at 72°C. After all 30 cycles, the samples were held at 72°C for an additional 5 minutes prior to cooling to 4°C. The PCR products were analyzed by agarose gel electrophoresis.

DNA hybridization analysis

Approximately 5µg of chromosomal DNA was digested with *Hind*III according to the manufacturer′s instructions (Promega, Inc., Madison, WI). The digested DNA was electrophoresesed at 35 mV overnight in a 0.8% agarose gels and then vacuum-blotted onto Nytran membranes (Schleicher & Schuell, Keene, NH).

Labeling of oligonucleotide with and detection of probe-target hybrids were both performed with the Genius System according to the manufacturer′s instructions (Mannheim, Indianapolis, IN). All hybridizations were done for 18 hours at 42°C without formamide. By assuming that 1% base-pair mismatching results in a 1°C decrease in *Tm* designations of "high" and "low" stringency were defined by salt concentration and temperature of post-hybridization washes. Homology between probe and target sequences was derived using calculated *Tm* the established method. High stringency is defined as 90% or greater homology, and low stringency is 80-85% sequence homology.

Table 17. Hybridization of oligonucleotides with HindIII restriction fragments of Rx1 DNA.

Oligonucleotide	Region	Stringency	
		Low	High
LSM12	Leader	N.D.	4.0, 9.1
LSM5	α-helix	N.D.	4.0
LSM3	α-helix	4.0, 9.1	4.0
LSM4	α-helix	4.0	4.0
LSM7	Proline	4.0, 9.1	4.0, 9.1
LSM1	Proline	4.0, 9.1	4.0, 9.1
LSM10	Repeats	N.D.	4.0, 9.1
LSM2	C-terminus	4.0, 9.1	4.0

Note. Values indicated are the sizes of restriction fragments expressed as kb.

Table 18. Summary of Hybridization at High and Low Stringency of 8 Oligonucleotides with HindIII Restriction Fragments of the 17 Pneumococcal Isolates Listed in Figure 2

Oligonucleotide	Percent with ≥1 band		Percent with ≥2 bands		Percent with 1 band		1 band/ ≥1 band	
	Low	High	Low	High	Low	High	Low	High
LSM12		82		59		24		0.29
LSM5		29		18		12		0.40
LSM3	65	35	41	0	24	35	0.36	1.00
LSM4	35	29	0	0	35	29	1.00	1.00
LSM7	94	71	71	41	24	29	0.25	0.42
LSM1	100	65	53	12	47	53	0.47	0.82
LSM10		94		59		35		0.37
LSM2	88	53	41	12	47	41	0.53	0.78

Note, for all values listed all 17 strains were examined.
If no value is listed, then no strains were examined.

Table 19. Amplification of Pneumococcal Isolates using the Indicated 5′ Prime Combination with the 3′ Primer LSM2 at the 3′ end of *pspA*

5' Primer	Region	Nucleotide Position			Amplified/ Tested	Percent Amplified
LSM8	- 35	47	to	70	2/14	14
LSM12	leader	162	to	188	8/14	57
LSM3	α-helical	576	to	598	3/14	21
LSM7	proline	1093	to	1117	12/14	86
LSM1	proline	1312	to	1331	14/14	100

Note, by 2x5 chi square analysis the different primers amplified different amplified different frequencies of pspAs ($P < 0.0001$). The tendency for there to be more amplification with the 3' most primers was significant at $P < 0.0001$.

Table 20. Size of amplified *pspA* fragments in kilobases

pspA Region	Primer Pairs	number of *pspAs* examined	Size	Range	S.D.
Full length	LSM12 + LSM2	9	1.9 - 2.3	0.4	0.17
α-helical	LSM12 + LSM6	6	1.1 - 1.5	0.4	0.17
Proline	LSM7 + LSM9	3	0.23	0	0
Repeats	LSM1 + LSM2	19	0.6 - 0.65	0.05	0.01

Note: amplification was attempted with each set of primers on a panel of 19 different pspAs. Data is shown only for *pspAs* that could be amplified with the indicated primer pairs.

Table 21 Pneumococcal Strains

Strain	Relevant characteristics
WU2	Capsular type 3, PspA type 1
D39	Capsular type 2, PspA type 25
R36A	Nonencapsulated mutant of D39, PspA type 25
Rx1	Nonencapsulated variant of R36A, PspA type 25
DBL5	Capsular type 5, PspA type 33
DBL6A	Capsular type 6A, PspA type 19
A66	Capsular type 3, PspA type 13
AC94	Capsular type 9L, PspA type 0
AC17	Capsular type 9L, PspA type 0
AC40	Capsular type 9L, PspA type 0
AC107	Capsular type 9V, PspA type 0
AC100	Capsular type 9V, PspA type 0
AC140	Capsular type 9N, PspA type 18
D109-1B	Capsular type 23, PspA type 12
BG9709	Capsular type 9, PspA type 0
BG58C	Capsular type 6A, PspA type ND
L81905	Capsular type 4, PspA type 25
L81905	Capsular type 4, PspA type 25
L82233	Capsular type 14, PspA type 0
L82006	Capsular type 1, PspA type 0

Table 22 PCR Primers

Primer	Sequence (5' to 3')
LSM1	CCGGATCCAGCTCCTGCACCAAAAAC
LSM2	GCGCGTCGACGGCTTAAACCCATTCACCATTGG
LSM3	CCGGATCCTGAGCCAGAGCAGTTGGCTG
LSM4	CCGGATCCGCTCAAAGAGATTGATGAGTCTG
LSM5	GCGGATCCCGTAGCCAGTCAGTCTAAAGCTG
LSM6	CTGAGTCGACTGGAGTTTCTGGAGCTGGAGC
LSM7	CCGGATCCAGCTCCAGCTCCAGAAACTCCAG
LSM8	GCGGATCCTTGACCAATATTTACGGAGGAGGC
LSM9	GTTTTTGGTGCAGGAGCTGG
LSM10	GCTATGGCTACAGGTTG
LSM11	CCACCTGTAGCCATAGC
LSM12	CCGGATCCAGCGTGCCTATCTTAGGGGCTGGTT
LSM13	GCAAGCTTATGATATAGAAATTTGTAAC

Table 23 Hybridization at high strength of eight different PspA probes with *Hind* III digests of 18 strains of *Streptococcus pneumoniae*

Probe	Strain																
	Rx1/ D39	WU2	DBL5	DBL6A	A66	AC94	AC17	AC40	AC107	AC100	AC140	DC109	BG9709	BG58C	L81905	L82233	L82006
FL-Rx1	4.0,9.1	3.8	3.7,5.8	3.0,3.4	3.6, 4.3	3.6,6.3	3.6,6.3	3.2, 3.6	3.6,6.3	4.0,8.0	3.0,4.0	3.3,4.7	2.2, 9.6	1.4,3.2, 3.6	3.6,5.2	3.7 8.2	4.3, 6.4
LSM12	4.0,9.1	3.8	3.7,5.8	3.0,3.4	4.3		3.6,6.3	3.2, 3.6		4.0,8.0	4.0	3.3,4.7	2.2, 9.6	1.4,3.2, 3.6	3.6	1.3,3.7	
LSM5	4.0					3.6,6.3							2.2, 9.6	3.6	1.2,2.3, 3.6		
LSM3	4.0	3.8				6.3							2.2	3.6	3.6		
LSM4	4.0												2.2	3.6	3.6	3.7	
LSM7	4.0,9.1	3.8	3.7	3.0,3.4	3.6			3.2, 3.6			3.0,4.0	3.3,4.7	2.2, 9.6	3.6	2.3 3.6	3.7	
LSM1	4.0,9.1	3.8	3.7,5.8	3.4		6.3		3.2	3.6	4.0	4.0		2.2		5.2		
LSM10	4.0,9.1	3.8	3.7	3.4	3.6, 4.3		3.6,6.3	3.2	3.6,6.3	4.0	4.0	3.3,4.7	2.2, 9.6	3.2 3.6	3.6,5.2	1.3,3.7	4.3, 6.4
LSM2	4.0		3.7			3.6	3.6		3.6,6.3	4.0	3.0,4.0	4.7					4.3

Note: All probes were tested versus *Hind*III digests of all strains. If no bands are listed none were detected. Strains Rx1 and D39 gave identical results and are shown in a single column. The full name of strain AC109 is AC109-1B

EXAMPLE 6 - RESTRICTION FRAGMENT LENGTH POLYMORPHISMS OF *pspA* REVEALS GROUPING

Pneumococcal surface A (PspA) is a protection eliciting protein of *Streptococcus pneumoniae*. The deduced amino acid sequence of PspA predicts three distinct domains; an α helical coiled-coil region, followed by two adjacent proline-rich regions, and ten 20 amino acid repeats. Almost all PspA molecules are cross-reactive with each other in variable degrees. However, using a panel of monoclonal antibodies specific for individual epitopes, this protein has been shown to exhibit considerable variability even within strains of the same capsular type. Oligonucleotide primers based on the sequence of *pspA* from *S. pneumoniae* Rx1 were used to amplify the full-length *pspA* gene and the 5′ portion of the gene including the α-helical and the proline-rich region. PCR-amplified product were digested with *Hha* I or *Sau*3A I to visualize restriction fragment length polymorphism of *pspA*. Although strains were collected from around the world and represented 21 different capsular types, isolates could be grouped into 17 families or subfamilies based on their RFLP pattern. The validity of this approach was confirmed by demonstrating that *pspA* of individual strains which are known to be clonally related were always found within a single *pspA* family.

Numerous techniques have been employed in epidemiological surveillance of pneumococci which include serotyping, ribotyping, pulsed field electrophoresis, multilocus enzyme electrophoresis, penicillin-binding protein patterns, and DNA fingerprinting. Previous studies have also utilized the variability of pneumococcal surface protein A (PspA) to differentiate pneumococci. This protein, which can elicit protective antipneumococcal antibodies, is a virulence factor found on all pneumococcal isolates. Although PspA molecules are commonly cross-reactive, they are seldom antigenically identical. This surface protein is the most serologically diverse protein know on pneumococci; therefore, it is an excellent market to be used to follow individual strains. Variations in PspA and the DNA surrounding its structural gene have proven useful for differentiation of *S. pneumoniae*.

When polyclonal sera are used to identify PspA, cross-reaction is observed between virtually all isolates. Conversely, when panels of monoclonal antibodies are used to compare PspA of independent isolation they are almost always observed to express different combinations of PspA epitopes. A typing system based on this approach has limitations because it does not easily account for differences in monoclonal binding strength to different PspA molecules. Moreover, some strains are weakly reactive with individual monoclonal antibodies and may not always give consistent results.

A less ambiguous typing system that takes advantage of the diversity of PspA was therefore necessary to develop and was used to examine the clonality of strains. This method involves examination of the DNA within and adjacent to the pspA locus. Southern hybridizations of pneumococcal chromosomal DNA digested with various endonucleases, such as *Hin*d III, *Dra* I, or *Kpn* I, and probed with labeled *pspA* provided a means to study the variability of the chromosome surrounding *pspA*. When genomic DNA is probed, the *pspA* and the *pspA*-like loci are revealed. In most digests the *pspA* probe hybridizes to 2-3 fragments and, digests of independent isolates were generally dissimilar.

Like the monoclonal typing system, the Southern hybridization procedure permitted the detection of clones of pneumococci. However, it did not provide a molecular approach for following *pspA* diversity. Many of the restriction sites defining the restriction fragment length polymorphism (RFLP) were outside of the *pspA* gene, and it was difficult to differentiate the *pspA* gene from the *pspA*-like locus. In an effort to develop a system to follow *pspA* diversity Applicants examined the RFLP of PCR-amplified *pspA*. Amplified *pspA* was digested with *Sau*3A I and *Hha* I, restriction enzymes with four base recognition sites. To evaluate the utility of this approach *pspA* from clinical and laboratory strains known to be clonally related as well as random isolates were examined.

Bacterial strains

Derivatives of the *S. pneumoniae* D39-Rx1 family were kindly provided by Rob Massure and Sanford Lacks (Figure 8). Eight clinical isolates from Spain and four isolates from Hungary, a gift from Alexander Tomasz. Seventy-five random clinical isolates from Alabams, Sweden, Alaska, and Canada were also studied.

PCR amplifications

The oligonucleotide primers used in this study are listed in Table 24. Chromosomal DNA, which was isolated according to procedures described by Dillard et al., was used as template for the PCR reactions. Amplification was accomplished in a 50 μl reaction containing approximately 50 ng template DNA, .25U Taq, 50 μM of each primer, 175 μM $MgCl_2$, and 200 μM dNTP in a reaction buffer containing 10 μM Tris-HCl, pH 9.0, 50μM KCl, 0.1% Triton X-100, 0.01% wt/vol. gelatin. The mixture was overlaid with mineral oil, and placed in a DNA thermal cycler. The amplification program consisted on an initial denaturation step at 94°C, followed by 29 cycles opf 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min. The final cycle included an incubation at 72°C for 5 min.

Restriction fragment analysis of PCR-amplified product

Aliquots of the PCR mixtures were digested with *Hha* I or *Sau*3A I in a final volume of 20μl according to manufacturer's protocols. After digestion the DNA fragments were electrophoresed on a 1.3% TBE agarose gel and stained with ethidium bromide. Fragment sizes were estimated by comparison to a 1kb DNA ladder (Gibco BRL).

Because of the variability of *pspA*, and the fact that the entire *pspA* sequence is known for only one gene, it has not been possible to design primers which amplify *pspA* from 100% of pneumococcal strains. However, oligonucleotide primers, LSM2 and LSM1, can amplify an 800 bp region of the C-terminal end in 72 of the 72 stains tested. Based on hybridizations at different stringencies, this region was found to be relatively conserved in pneumococcal strains, and thus would not be expected to be optimal for following restriction polymorphisms within the *pspA* molecule. LSM13 and LSM2, primers which amplify the full length *pspA* gene, can amplify *pspA* from approximately 79% 55/75 of the strains tested (Table 25).

Stability of amplified RFLP pattern within clonally related pneumococci

To determine the stability of *pspA* during long passages in vitro, we examined the RFLP pattern of the *pspA* gene of the derivatives of the *S. pneumoniae* D39-Rx1 family. Rx1 is an acapsular derivative of *S. pneumoniae* D39, the prototypical pneumococcal laboratory strain isolated by Avery in 1914. Throughout the 1900's spontaneous and chemical mutations have been introduced into D39 by different laboratories (Figure 8). During this period unencapsulated strains were maintained in vitro, and D39 was passed both in vivo and in vitro passage. All the derivatives of D39, including Rx1, R6, RNC, and R36A, produced a 1.9 kb fragment upon PCR amplification of full length *pspA*. All members of the family exhibited the RFLP pattern. Digestion with *Sau3A* I of PCR amplified full length *pspA* revealed a .83, .58, .36 and a .27 kb fragment in all of the D39-rX1 derivatives of the family. Digesting the full length *pspA* with *Hha* I resulted in bands which were .76. .47, .39, .35, and .12 kb (Figure 9 or Table 26).

The stability of *pspA* polymorphism was also investigated using pneumococcal isolates which had previously been shown to be clonally related by other criteria, including capsule type, antibiotic resistance, enzyme electromorph, and PspA serotype. Three sets of isolates, all of which were highly penicillin resistant, were collected from patients during an outbreak in Hungary and two separate outbreaks in Spain. PCR amplified full length *pspA* from the capsular type 19A pneumococcal strains from the outbreak in Hungary, DB18, DB19, DB20, and DB21, resulted in a band approximately 2.0 kb. After digesting full length *pspA* with *Hha* I, four fragments were visualized., 89, .48, and .28 kb. Digestion with *Sau*3A I yielded five fragments .880, .75, .35, .34, and .10kb. Capsule type 6B pneumococcal strains, DB1, DB2, DB3, and DB4, were obtained from an outbreak in Spain. Full length *pspA* from these strains were approximately 1.9 kb. Digestion of the PCR-amplified fragment with *Hhs* I resulted in four fragments which were .83, .43, .33, and .28 kb. *Sau*3A I digestion yield a .88, .75, .34, and .10 kg fragments. DB6, DB8, and DB9, which are capsular serotype 23F strains, were isolated from a second outbreak in Spain. DB6, DB8, and DB9 had an amplified *pspA* product which was 2.0 kb. *Hha* I digested fragments were .90, .52, .34, and .30 kb and *Sau*3A I fragments were .75, .52, .39, .22, .20, and .10 kb in size (Figure 10). DB7 had a 19A capsular serotype and was not identical to DB6, DB8, and DB9. In the D39/Rx1 family and in each of the three outbreak families the size of the fragments obtained from the *Hha* I and the *Sau*3A I digests totaled approximately 2.0 kb which is expected if the amplified product represents a single *pspA* sequence.

Diversity of RFLP pattern of amplified pspA from random pneumococcal isolates

PCR amplification of the *pspA* gene from 70 random clinical pneumococcal isolates yielded full-length *pspA* ranging in size from 1.8 kb to 2.3 kb. RFLP analysis of PCR-derived *pspA* revealed two to six DNA fragments ranging in size from 100 bp to 1.9 kb depending on the strain. The calculated sum of the fragments never exceeded the size of the original amplified fragment. Not all pneumococcal strains had a unique *pspA*, and some seemingly unrelated isolates from different geographical regions and different capsular types exhibited similar RFLP patterns. Isolates were grouped into families based on the number of fragments produced by *Hha* I and *Sau*3A I digests and the relative size of these fragments.

Based on the RFLP patterns it was possible to identify 17 families with four of the families containing pairs of subfamilies. Within families all of the restriction fragments were essentially the same regardless which restriction enzyme was used. The subfamilies represent situations where two families share most but not all the restriction fragments. With certain strains an FRLP pattern was observed where detectable fragment size differed from the pattern of the established family by less than 100 bp. Since the differences were considered small compared to the differences in the fragment size and the number of fragments between families, they were not considered in family designation. The RFLP pattern of two isolates from six of the families is pictured in Figure 11, Table 27. These families were completely independent of the capsular type or the protein type as identified by monoclonal antibodies (Table 28 and 29).

Previous DNA hybridization studies have demonstrated that the *pspA* gene of different isolates are the most conserved in their 3′ region of the gene and more variable in the 5′ region of the gene. Thus, if seemed likely that the differences in the *pspA* families reflected primarily differences in the 5′ end of the gene. To confirm this theory, the α helical and proline region of *pspA* was examined without the amino acid repeats. Nucleotide primers LSM13 and KSH2 were used to amplify this fragment which is approximately 1.6 kb. Examination of this region of *pspA* afforded two things.

This primer pair permitted amplification of 90% of the strains which is greater than the 75% of the strains which can be amplified with oligonucleotides which amplify the full length gene. Second, it allowed Applicants to examine if the original groupings which were based on the full length gene coincide with the fingerprint patterns obtained by looking at the 5′ half of the gene.

Figure 12 contains the same strains which were examined in Figure 11 but the PCR products were amplified with SKH2 and LSM13. The RFLP patterns obtained from digestion of the amplified α helical and proline rich region confirms the original designated families. However, these primers amplify a smaller portion of the *psaA* and therefore the difference is the families is not as dramatic as the RFLP patterns obtained from the RFLP pattern of the full length gene.

The polymerase chain reaction has simplified the process of analyzing *pspA* gene and have provided a means of using *pspA* diversity to examine the epidemiology of *S. pneumoniae*. Because not all strains contained a unique fingerprint of *pspA*, RFLP patterns of *pspA* cannot be used alone to identify the clonality of a strain. These results indicate the RFLP of PCR-amplified *pspA* from pneumococcal strains in conjunction with other techniques may be useful for identifying the clonal relatedness among pneumococcal isolates, and that this pattern is stable over long passages in vitro.

These findings suggests that the population of *pspA* is not as diverse as originally believed. PCR-RFLP of *pspA* may perhaps represent a relatively simplistic technique to quickly access the variability of the gene within a population. Further, these findings enable techniques to diagnose. *S. pneumoniae* via PCR or hybridization by primers on probes to regions of *pspA* common within groupings.

The sequence studies divide the known strains into several families based on sequence homologies. Sequence data demonstrates that there have been extensive recombinations occurring in nature within *pspA* genes. The net effect of the recombination is that the "families" identified by specific sequences differ depending upon which part of the *pspA* molecule is used for analysis. "Families" or "grouping identified by the 5′ half of the alpha-helical region, the 3′ half of the α-helical region and the proline rich region are each distinct and differ slightly from each other. In addition there is considerable evidence of other diversity (including base substitute and deletions and insertions in the sequences) among otherwise closely related molecules.

This result indicates that it is expected that there will be a continuum of overlapping sequences of PspAs, rather than a discrete set of sequences.

The findings indicate that there is the greatest conservation of sequence in the 3′ half of a α-helical region and in the immediate 5′ tip. Because the diversity in the mid half of the α-helical region is greater, this region is of little use in predicting cross-reactivity among vaccine components and challenge strains. Thus, the sequence of 3′ half of the alpha-helical region and the 5′ tip of the coding sequence are likely to the critical sequence for predicting PspA cross-reactions and vaccine composition.

The sequence of the proline-rich region may not be particularly important to composition of a vaccine because this region has not been shown to be able to elicit cross-protection even though it is highly conserved. The reason for this is presumably because antibodies to epitopes in this region are not surface exposed.

Based on our present sequences of 27 diverse *pspAs* we have found that there are 4 families of the 3′ half of the α-helical region and 2-3 families of the very 5′ tip the α-helical region. Together these form 6 combinations of the 3′ and 5′ families. This approach therefore should permit us to identify a panel of *pspAs* with 3′ and 5 helical sequences representative of the greatest number of different *pspAs*. See Fig. 13.

Table 29. Relationship of Capsular type and RFLP family.

RELATIONSHIP BETWEEN CAPSULAR TYPE AND RFLP FAMILY																											
	Capsule Type																										
pspA family	1	2	3	4	5	6	6A	6B	7	8	9A	9L	9N	9V	10	11	12	13	14	15	19	22	23	31	33	35	ND
A			3																								
B			1	1																							
C						2	1	2														2					1
D			1				1																				
DD				2																							
E			1	2		1																					
F						1											1						3				1
FF			1			1									1	1											
G																1					1						
H			1			1			2	1							1		1	1	1						
I											2		2	4													
II					1																						
J	2					2						1	1					1	2		2						1
K		1																					1		1		
KK	1					1		1											1			1					
L																							1				1
M								1																1			
MM																					1						

Table 24. Oligonucleotides used in this study.

Designation	Sequence 5'-3'	Nucleotide position
LSM2	GCG CGT CGA CGG CTT AAA CCC ATT CAC CAT TGG	1990 to 1967
LSM1	CCG GAT CCA GCT CCT GCA CCA AAA AC	1312 to 1331
LSM13	GCA AGC TTA TGA TAT AgA ATT TTG TAA C	1 to 26
SKH2	CCA CAT ACC GTT TTC TTG TTT CCA GCC	1333 to 1355

Table 25. Amplification of *pspA* from a panel of 72 independent isolates* of *S. pneumoniae*.

CAPSULE TYPE	NUMBER OF STRAINS EXAMINED	LSM13 AND LSM2	LSM13 AND SKH2
		% OF STRAINS AMPLIFIED	% OF STAINS AMPLIFIED
1	3	100	100
2	1	100	100
3	8	50	87
4	6	67	100
5	1	100	100
6	7	29	86
6A	2	100	100
6B	6	100	100
7	2	50	100
8	1	100	100
9V	3	100	100
9A	2	100	100
9L	1	100	100
9N	3	100	100
10	1	100	100
11	2	50	100
12	2	0	100
13	1	100	100
14	4	0	75
15	2	50	50
19	5	100	100
22	3	33	100
23	1	100	100
33	1	0	100
35	1	0	100
nd	3	100	100

* Our strain collection contains several groups of isolates known to be previously to be clonal and collected for that purpose. The data reported in the table includes only representative isolate from such clonal groups.

Table 26. Rx1-D39 derivatives

ISOLATE	SIZE OF *HhA* I DIGESTS	SIZE OF *Sau*3A I DIGESTS (Kb)
D39	.76, .47, .39, .35, .12	.83, .58, .36, .27
Rx1	.76, .47, .39, .35, .12	.83, .58, .36, .27
R800	.76, .47, .39, .35, .12	.83, .58, .36, .27
R6	.76, .47, .39, .35, .12	.83, .58, .36, .27
R61	.76, .47, .39, .35, .12	.83, .58, .36, .27
R6X	.76, .47, .39, .35, .12	.83, .58, .36, .27
R36NC	.76, .47, .39, .35, .12	.83, .58, .36, .27
R36A	.76, .47, .39, .35, .12	.83, .58, .36, .27

Table 27. Strain information and family designation of independent isolates.

STRAIN	CAPSULE TYPE	PspA TYPE	FAMILY	SIZE OF *Hha* I FRAGMENTS	SIZE OF *Sau*3A I FRAGMENTS
BG9163	6B	21	C	1.55, .35	1.05, .35, .22
EF6796	6A	1	C	1.5, .35	1.05, .35, .22
EF5668	4	12	DD	1.25, .49, .32	1.0, .80, .35
EF8616A	4	ND	DD	1.25, .49, .32	1.0, .80, .35
EF3296	4	20	E	1.0, .40, .33	1.15, .50, .34
EF4135	4	ND	E	1.0, .40, .33	1.15, .50, .34
BG7619	10	ND	F	1.3, .40, .29, .10	.82, .76, .35
BG7941	11	ND	F	1.3, .40, .29, .10	.82, .76, .35
BG7813	14	8	H	1.05, .70, .36	.90, .77, .35
BG7736	8	ND	H	1.05, .70, .36	.90, .77, .35
AC113	9A	ND	I	1.4, .34, .28	1.2, .80
AC99	9V	5	I	1.4, .34, .28	1.2, .80

Table 28. Relationship of RFLP family and PspA type.

RELATIONSHIP BETWEEN PSPA TYPE AND RFLP FAMILY																					
	PspA Type																				
pspA FAMILY	0	1	3	5	8	12	13	16	18	19	20	21	24	25	26	30	33	34	36	37	ND
A		1					1														
B														1	1						1
C		2										1	1								4
D								1		1											
DD						2															
E			1								1					1					
F						1											1				4
FF														1							3
G								1											1		
H	1				1			1		1											5
I	3			1				2	2												1
II																	1				
J	4								1	1										1	3
K	1									1				1							
KK	1																			1	3
L								1													1
M								1	1									1			1
MM						1															

EXAMPLE 7 - ABILITY OF PspA IMMUNOGENS TO PROTECT AGAINST INDIVIDUAL CHALLENGE STRAINS

CBA/N or BALB cJ mice were given 1 injection of 0.5 - μg PspA in CFA, followed 2 weeks later by a boost in saline, and challenged between 7 and 14 (average 10) days post boost. Control mice were administered a similar immunization regimen, except that the immunization came from an isogeneic strain unable to make PspA. The PspA was either full length, isolated from pneumococci or cloned full length or BC100 PspA, as little statistical significance has been seen in immunogenicity between full length PspA and BC100. The challenge doses ranged from about 10^3 to 10^4 pneunocci in inoculum, but in all cases the challenge was at least 100 times LD_{50}.

The results are shown in the following Tables 30 to 60, and the conclusions set forth therein.

From the data, it appears that an antigenic, immunological or vaccine composition can contain any two to seven, preferably three to five PspA, e.g., PspAs from R36A and BG9739, alone, or combined with any or all of PspAs from Wu2, Ef5668, and DB15. Note that surprisingly WU2 PspA provided better protection against D39 that did R36a/Rx1/D39, and that also surprisingly PspA from Wu2 protected better against BG9739 than did PspA from BG9739. Combinations containing R36A, BG9739 and WU2 PspAs were most widely protective; and therefore, a preferred composition can contain any three PspA, preferably R36A, BG9739 and WU2. The data in this Example shows that PspA from varying strains is protective, and that it is possible to formulate protective compositions using any PspA or any combination of the PspAs from the eight different PspAs employed in the tests. Similarly, one can select PspAs on the basis of the groupings in the previous Example. Note additionally that each of PspA from R36A, BG9739, EF5668 and DBL5 are, from the data, good for use in compositions.

A note about use of medians rather than averages. Applicants have chosen to express data as median (a non-parametric parameter) rather than averages because the times to death do not follow a normal distribution. In fact there are generally two peaks. One is around day 3 or 6 when most of the mice die and the other is at > 21 for mice that live. Thus, it becomes nonsensical to average values like 21 or 22 with values like 3 or 6. One mouse that lives out of 5 has a tremendous effect on such an average but very little effect on the median. Thus, the median becomes the most robust estimator of time to death of most of the mice.

TABLE 30

Relative ability of different PspAs to Protect against each challenge strains of *S. pneumoniae*
(Summary of statistically significant protection)

			Vaccine PspA										
Challenge	Caps	PspA	*pspA*	R36A, Rx1, D39	JD908/WU2	JS1020/BG 9739	EF3296	EF5668	L81905	JS5010.3 DBL5	JS3020 DBL6A	All Immune	best protect
Strain	type	type	family	K	a	b	E	DD	b	II	D	-	-
D39	2	25	K	++	+++			+				++	+++
WU2	3	1	a	+++	+++	+++		+++	+++	+++	+++	+++	+++
A66	3	13	a	+++	+++	+++		+++	+++	+++	+±	+++	+++
EF10197	3	18	M	+++		+++						+++	+++
ATCC6303	3	7	a	+++								+++	+++
BG9739	4	26	b	+	+++	+	0+	0	+±	0	0	++	+++
EF3296	4	20	E	+±	+±	0+				0	0	0	+±
EF5668	4	12	DD	+	0	+++	0+	+++	0+	+	0+	++	+++
L81905	4	23	b	+	+	++	++	0	+	+±	+±	++	++
DBL5	5	33	II	+		+		+	+	++	0	++	++
EF6796	6A	1	C	+++								+++	+++
DBL6A	6A	19	D	+++	+±	++	+±	+++	+±	+±	+++	++	+++
BG9163	6B	21	C	+++		+++						+++	+++
BG7322	6B	24	C	+++	+++	+±	0	+++	+±	+++	+±	+++	+++

Note: Empty cells indicate that no experiment has been done. Bold means significant at $P < 0.05$, Small font bold (+) means $0.02 \leq P < 0.05$. Large font bold means $P < 0.02$. For this table statistical significance refers to delay in time to death except as indicate in the (+) footnote below. When "all immune" showed significant protection against death but individual data cells did not, the result for "all immune" is presented under best protection on the assumption that if more mice were done in each data cell one or more of them would have exhibited significant protection against death.

+++ = statistically significant protection against death; ≥ 50% protection from death

++ = statistically significant protection against death; < 50% protection from death

+± = statistically significant delay in death; ≥ protection from death

\+ = statistically significant delay in death; <20 protection from death, (or significant protection against death but not a significant delay in death)

0++ = Not statistically delay in time to death; but ≥ 50% protection from death

0+ = Not statistically delay in time to death; but > 1.5 day extension in median time to death or ≥ 20% protection from death.

0 = No apparent extension in time to death or protection from death.

TABLE 31

Relative ability of different PspAs to Protect against each challenge strains of *S. pneumoniae* (Expressed as Median days Alive post challenge)													
			Vaccine PspA										
Challenge	Caps	PspA	*pspA*	R36A, Rx1, D39	JD908/WU2	JS1020/BG 9739	EF3296	EF5668	L81905	JS5010.3 DBL5	JS3020 DBL6A	All immune	All control
Strain	type	type	family	K	a	b	E	DD	b	II	D	–	–
D39	2	25	K	4.5	>21			4				5	2
WU2	3	1	a	>21	>21	>21		>21	>21	>21	>21	>21	2
A66	3	13	a	>21	>21	>21		>21	>21	>21	4	>21	2
EF10197	3	18	M	>21		>21						>21	2
ATCC6303	3	7	a	>21								>21	5
BG9739	4	26	b	3	>21	6	3	3	5,13	2	2	3	2
EF3296	4	20	e	5	5	4.5				2	2	3	2
EF5668	4	12	DD	6	2	>21	13	>21	4	>21	5	8	3
L81905	4	23	b	5	5	8	6	3	5	3	3.5	5	2
DBL5	5	33	II	4		3		3	3.5	6	2	3.5	2
EF6796	6A	1	C	>21								>21	1
DBL6A	6A	19	D	>21	8.5	13	9	>21	8	12	>21	12.5	5.5
BG9163	6B	21	C	>21		>21						>21	8.5
BG7322	6B	24	C	>21	>21	14.5	6	>21	12.5	>21	11	>21	7

Note: Bold denotes statistically significant extension of life at $P < 0.05$. **Small font denotes** $0.02 \leq P < 0.05$; **large font denotes** $P < 0.02$. Median times to death indicated as 8, >21, are situations where the medium as not within a continuum of values. In those cases the numbers shown are those closest to the median. In these cases the values give are those closest to the calculated median. Fractional values such as 3.5, indicate that the median is halfway between two numbers, in this case 3 and 4. As indicated in the original data (S103B), some experiments were terminated prior to 21 days post infection. There is little reason to assume, however, that results would have been significantly effected by the early termination's since very few mice infected with the strains used in those studies, have ever been observed to die later than 10 or 15 days post challenge. For statistical purposes all mice alive at the end of experiments were assumed to have been completely protected, and for the sake of calculations all surviving mice were assigned values of >21.

TABLE 32

Ability of different PspAs to Protect Against Each Challenge strain of *S. pneumoniae*
(Expressed as increase in survival time in days)
(A denotes ≥ 50% immune mice alive)

			Vaccine PspA									
Challenge	Caps	PspA	*pspA*	R36A, Rx1, D39	JD908/WU2	JS1020/ BG9739	EF3296	EF5668	L81905	JS5010.3 DBL5	JS3020 DBL6A	All immune	Best Result
Strain	type	type	family	K	a	b	E	DD	b	II	D		
D39	2	25	K	2.5	A			2				3	A
WU2	3	1	a	A	A	A		A	A	A	2	A	A
A66	3	13	a	A	A	A		A	A	A	2	A	A
EF10197	3	18	M	A		A						A	A
ATCC6303	3	7	a	A								A	A
BG9739	4	26	b	1	A	4	1	1	3,11	0	0	1	A
EF3296	4	20	E	3	3	2.5				0	0	1	3
EF5668	4	12	DD	3	-1	A	10	A	1	A	2	5	A
L81905	4	23	b	3	3	6	4	1	3	1	1.5	3	6
DBL5	5	33	II	2		1		1	1.5	4	0	1.5	4
EF6795	6A	1	C	A								A	A
DBL6A	6A	19	D	A	3	7.5	3.5	A	2.5	6.5	A	7	A
BG9163	6B	21	C	A		A						A	A
BG7322	6B	24	C	A	A	7.5	-1	A	5.5	A	4	A	A
				R36A	WU2	BG9739	EF3296	EF5668	L81905	DBL5	DBL6A	A11	Best

Note: Bold denotes statistically significant extension of life at P < 0.05 Small font denotes $0.02 \leq P < 0.05$; large font denotes P < 0.02. Median increases in survival listed as 3, 9 or 1,A denote groups where the median does not fall within a continuum of values. In these case the values give are those closest to the calculated median. Fractional values such as 3.5, indicate that the median is halfway between two numbers, in this case 3 and 4.

TABLE 33

Relative ability of different PspAs to Protect against each challenge strains of *S. pneumoniae*
(expressed % alive at 21 days post challenge)

			Vaccine PspA										
Challenge	Caps	PspA	*pspA*	R36A, Rx1, D39	JD908/WU2	JS1020/ BG9739	EF3296	EF5668	L81905	JS5010.3 DBL5	JS3020 DBL6A	All immune	All control
Strain	type	type	family	K	a	b	E	DD	b	II	D	–	
D39	2	25	K	38	60			30				38	3
WU2	3	1	a	100	100	100		100	100	100	100	100	1.5
A66	3	13	a	75	100	80		75	100	60	20	76	5
EF10197	3	18	M	100		80						90	0
ATCC6303	3	7	a	100								100	0
BG9739	4	26	b	11	60	13	25	0	25	0	0	12	0
EF3296	4	20	E	25	20	10				0	0	8	0
EF5668	4	12	DD	22	25	60	40	100	40	60	0	41	9
L81905	4	23	b	10	0	31	40	0	0	14	0	14	0
DBL5	5	33	II	10		14		0	0	29	0	4	0
EF6795	6A	1	C	100								100	0
DBL6A	6A	19	D	67	25	33	0	60	25	0	80	35	4
BG9163	6B	21	C	89				80				86	20
BG7322	6B	24	C	100	60	25	0	89	25	80	25	55	6

Bold, denotes statistically significant protection against death at $P < 0.05$. Bold small font, indicates significant protection against death at $0.02 \leq P$, 0.05. Bold large font, indicates significant protection against death at $P < 0.02$.

TABLE 34

Relative ability of different PspAs to Protect against each challenge strains of *S. pneumoniae*
(%protected from death at 21 days post challenge)

				Vaccine PspA									
Challenge	Caps	PspA	*pspA*	R36A, Rx1, D39	JD908/WU2	JS1020/ BG9739	EF3296	EF5668	L81905	JS5010.3 DBL5	JS3020 DBL6A	All immune	Best result
Strain	type	type	family	K	a	b	E	DD	b	II	D	--	
D39	2	25	K	36	59			28				36	59
WU2	3	1	a	100	100	100		100	100	100	100	100	100
A66	3	13	a	71	100	79		74	100	58	16	75	100
EF10197	3	18	M	100		80						90	100
ATCC6303	3	7	a	100								100	100
BG9739	4	26	b	11	60	13	25	0	25	0	0	12	60
EF3296	4	20	E	25	20	10				0	0	8	25
EF5668	4	12	DD	14	18	56	34	100	34	56	-10	35	100
L81905	4	23	b	10	0	31	40	0	0	14	0	14	40
DBL5	5	33	II	10		14		0	0	29	0	4	29
EF6796	6A	1	C	100								100	100
DBL6A	6A	19	D	66	22	30	-4	58	22	-4	79	33	79
BG9163	6B	21	C	86		75						83	86
BG7322	6B	24	C	100	57	22	0	88	22	79	22	52	100

Bold, denotes statistically significant protection against death at $P < 0.05$. Bold small font, indicates significant protection against death at $0.02 \le P < 0.05$. Bold large font, indicates significant protection against death at $P < 0.02$.

% protected has been corrected for any survivors in the control mice.

% protected = 100 x (% alive in immune - % alive in control)/(100-% alive in control). Thus, if there were any mice alive in the control animals, the calculated "% protected" is less then the observed "% alive" listed in the previous table. The only exceptions to this are if 100% of immunized mice lived. Negative numbers mean that less immunized mice lived then did control mice. Please note that none of these negative numbers are significant even though we are using a one tailed test.

TABLE 35

Recommended Immunogens to Protection against the indicated challenge strains of *S. pneumoniae* Based on Protection Score
Based on median days alive and percent protected
(numbers refer to preference as a vaccine strain with respect to the indicated challenge strain, 1=best)

				Vaccine PspA							
Challenge	Caps	PspA	*pspA*	R36A, Rx1, D39	JD908/WU2	JS1020/BG 9739	EF3296	EF5668	L81905	DBL5 JS5010.3	DBL6A JS3020
Strain	type	type	family	K	a	b	E	DD	b	II	D
D39	2	25	K	2	1			3			
WU2	3	1	a	1	1	1		1	1	1	1
A66	3	13	a	2	1	2		2	1	3	0
EF10197	3	18	M	1		2					
ATCC6303	3	7	a	1							
BG9739	4	26	b	3	1	2	3	3	2	0	0
EF3296	4	20	E	1	1	2				0	0
EF5668	4	12	DD	0	0	2	3	1	0	2	0
L81905	4	23	b	2	0	1	1	0	0	0	0
DBL5	5	33	II	2		3		0	3	1	0
EF6796	6A	1	C	1							
DBL6A	6A	19	D	2	0	3	0	2	0	0	1
BG9163	6B	21	C	1		1					
BG7322	6B	24	C	1	2	3		1	3	1	3
Number of #1's				7	5	3	1	3	2	3	2

Bold, denotes statistically significant protection against death at $P < 0.05$. Where more than one PspA were equally protective, the same values were given to each. Recommendations are based on days to death with % protection dividing ties, especially among those where greater than 50% of mice lived to 21 days. "0" indicates test were conducted but compared to the other PspAs this one is not recommended.

Conclusions: Statistically significant protection against death with > 50% protection; 11/14 of the strains = 79%
Statistically significant protection against death; 13/14 strains = 93%
Statistically significant extension of life in 14/14 or 100% of strains.

TABLE 36

Best Choice for Vaccine Components as of 95/8/27						
	Vaccine Component (cumulative strains protected) % maximally protected					
Criterion	1	2	3	4	5	6
≥#1 PspA for each challenge strain	R36A (7) 50%	WU2 (10) 71%	BG9739* (11) 79%	EF5668 (12) 86%	DBL5 (13) 93%	DBL6A (14) 100%
≥#2 PspA for each challenge strain	R36A (12) 86%	BG9739 (12) 100%				
Max score (+) type score	R36A (9) 64%	WU2 (11) 79%	BG9739 (13) 92%	DBL5 (14) 100%		
Max Increase in Days alive	R36A (9) 64%	WU2 (11) 79%	BG9739 (13) 92%	DBL5 (14) 100%		
% protected	R36A (7) 50%	WU2 (10) 64%	DBL5 (11) 79%	EF5668 (12) 86%	DBL6A (13) 92%	EF3296 (14) 100%
Theoretical mixture based on a few testable assumptions (see below)	R36A (10) 64%	BG9739 (12) 86%	DBL5 (13) 92%	EF3296 (14) 100%		

*This is not a unique combination. See table below.

TABLE 37

Combinations where all Challenge Strains have a Vaccine strain with a score of ≥ #2				
Number of PspAs in Combination	Combination	Number of #1 strains	Total #1s	Total #1s and #2s
2	R36A + BG9739	8	10	20
3	R36A + BG9739 + WU2	11	15	25
3	R36A + WU2 + DBL5	11	15	21
3	R36A + WU2 + EF5668	11	15	23
3	R36A + WU2 + DBL5	11	15	22

TABLE 38

Pooled Data for Protection against D39 by various PspAs;
Days alive for each mouse

			Days to Death/immunogen				
Exp.	Log CFU D39	Mice	Rx1/R36A D39	JD908 (WU2)	EF5668	All immune	control
143	4.5	CBA/N			1,1,2,2,2		1,1,2,2,3
E145	4.0	CBA/N	2,3,3,3,4				1,1,2,3,4
E028 BCG	5.93	BALB/c	3,3x >21				2,2,2,4
E143	3.0	CBA/N			2,6,3x>10		3,3,3,5,5
E140 BC100	2.81	CBA/N	4,4,5,7,15				2,2,2
E169	2.7	CBA/N	2,4x>21	2,5,3x>21			1,2,2,2,3
E154	2.6	CBA/N	2,2,3,2x>2 1				4x1, 6x2,3,3,4
All ≤3.0			2,3,3,3,4,4, 4,5,7,15		1,1,2,2,2		4x1, 6x2,3,3,4
All			4x2, 5x3, 3x4,5,7,15, 9x>21	2,5,3x>21	1,1,2,2,2,2,6, 3x>21	1,1,9x2,6x3,3 x4,5,5,6,7,15, 15x>21	5x1,16x2,6x3,4, 4,5,5,5,>21

TABLE 39

Pooled Data for Protection against D39 by various PspAs
Median Days Alive & alive:dead with corresponding *P* values

Exp.	Log CFU	Mice	Rx1/R36A D39		JD908 (WU2)		EF5668		All immune		Control	
	D39		med	a:d	med	a:d	med	a:d	med	a:d	med	a:d
143	4.5	CBA/N					2 n.s.	0:5			2	0:5
E145	4.0	CBA/N	3 n.s.	0:5							2	0:5
E028 BCG	5.93	BALB/c	>21 .029	3:1 n.s.							2	0:4
E143	3.0	CBA/N					>21 n.s.	3:2 n.s.			3	0:5
E140 BC100	2.81	CBA/N	5 0.018	0:5							2	0:3
E169	2.7	CBA/N	>21 .016	4:1 .024	>21 .016	3:2 n.s.					2	0:5
E154	2.6	CBA/N	3 n.s.	2:3 n.s.							2	1:5
All ≤3.0			4 .0008	0:10			2 n.s.	0:5			2	0:13
All			4.5 .0057	9:15 .001 ++	>21 .0006	3:2 .0045 +++	4 (2.6) n.s.	3:7 .034 +	5 .0001	15:24 .0002 ++	2	1:32
% alive				38		60		30		38		3
				36		59		28		36		
			Rx1/D39		WU2		EF5668		All immune		controls	

TABLE 40

Pooled Data for Protection against WU2. by various PspAs												
Exp.	CFU WU2	MICE	Days to Death/immunogen									
			FL-R36A	Rx1 BC100	JD108 (WU2)	JS1020 (BG9739)	BG9739 bc100	EF5668	L81905 bc100	DBL5 bc100	JS3020 (DBL6A)	control
Dr. Ed, expt.											+ + +	
lots of prior expts.			+ + +									
E012	3.0	CBA/N	15x>21									1,1,11x2,7x3,4
E028	6.01	BALB/c	4x>21 0.05/n.s.									4,6,6,>21
E084	3.75^1	CBA/N				3x>15						1,2,2,2,3,3,>15
E125 bc100	3.57	CBA/N					4x>21		4x>21	4x>21		2,2,3,3,3,>21
E129	3.18	CBA/N				5x>23						2,2,2,2,3
E140 BC100	3.43	CBA/N		4x>21								1,5x2,3,4
E143	3.0	CBA/N						8x>10				1,1,2,2,2,3
E144	3.9	CBA/N									5x>21	5x2
E172	3.98	CBA/N			5x>21							5x3
All			19x>21	4x>21	5x>21	8x>21	4x>21	8x>21	4x>21	4x>21	5x>21	6x1,33x2,20x3, 4,4,4,6,6,>21
All Immune			61x>21									

TABLE 41

Pooled Data for Protection against WU2 by various PspAs

Exp.	CFU WU2	MICE	Median days Alive – Alive:Dead – P value based on Alive:Dead P value calculated compared to pooled controls (in this case 65 control mice) SCORE									
			FL-R36A	Rx1 BC100	JD108 (WU2)	JS1020 (BG9739)	BG9739 bc100	EF5668	L81905 bc100	DBL5 bc100	JS3020 (DBL6A)	control
Dr. Ed, expt.											+++	
lots of prior expts.			+++									
E012	3.0	CBA/N	15x>21									1,1,11x2,7x3,4
E028	6.01	BALB/c	4x>21 0.05/n.s.									4,6,6,>21
E084	3.75[1]	CBA/N				3x>15						1,2,2,2,3,3,>15
E125 bc100	3.57	CBA/N					4x>21		4x>21	4x>21		2,2,3,3,3,>21
E129	3.18	CBA/N				5x>23						2,2,2,2,3
E140 BC100	3.43	CBA/N		4x>21								1,5x2,3,4
E143	3.0	CBA/N						8x>10				1,1,2,2,2,3
E144	3.9	CBA/N									5x>21	5x2
E172	3.98	CBA/N			5x>21							5x3
All			>21 19:0 <.0001 +++	>21 4:0 <.0001 +++	>21 5:0 <.0001 +++	>21 8:0 <.0001 +++	>21 4:0 <.0001 +++	>21 8:0 <.0001 +++	>21 4:0 <.0001 +++	>21 4:0 <.0001 +++	>21 4:0 <.0001 +++	2 1:64
% alive			100	100	100	100	100	100	100	100	100	2

Pooled Data for Protection against WU2 by various PspAs												
Exp.	CFU WU2	MICE	Median days Alive -- Alive:Dead -- P value based on Alive:Dead P value calculated compared to pooled controls (in this case 65 control mice) SCORE									
			FL-R36A	Rx1 BC100	JD108 (WU2)	JS1020 (BG9739)	BG9739 bc100	EF5668	L81905 bc100	DBL5 bc100	JS3020 (DBL6A)	control
			FL-R36A	Rx1 BC100	JD108 (WU2)	JS1020 (BG9739)	BG9739 bc100	EF5668	L81905 bc100	DBL5 bc100	JS3020 (DBL6A)	control

WU2 Challenge	days of death	median days of death	alive: dead	*P* value based on days to death	*P* value based on alive:dead	Score	% alive	% protected
All immune	61x>21	>21	61:0	<.0001	<.0001	+++	100	100
All controls	6x1,33x2,20 x3,4,4,4,6,6, >21	2	1:64				2	2

TABLE 42

Pooled Data for Protection against A66. by various PspAs

Exp.	CFU A66	MICE	Days to Death/immunogen											
			FL-R36A/ D39	Rx1 BC100	JD908 (WU2)	JS1020 (BG9739)	BG9739 bc100	EF5668	L81905 FL	L81905 bc100	JS5010.3 FL (DBL5)	DBL5 bc100	JS3020 (DBL6A)	control
E169	2.60	CBA/N	5x>21		5x>21									1,1,2,2, 6
E152 bc100	2.78	CBA/N					4x>21			4x>21		4x>21		3x2,3,6, 6,>21
E104	3.0	CBA/N				2,8,3x>22					3,4,4,2x >22		2,4,4,5, >22	2,2,2,2, 3
E143	3.0	CBA/N						4,4x>10						2,2,3,3
E140	3.43	CBA/N		4x>21										1,1,1
E172	3.94	CBA/N							5x>21					
E145	3.97	CBA/N	13,4x>21											1,2,2,2, 4
E121	4.16	CBA/N	3x3,2x4,5 x>21											1,8x2,> 21
All			3x3,2x4,1 3,14x>21	4x>21	5x>21	2,8,3x>21	4x>21	4,4x>21	5x>21	4x>21	3,4,4,2x >21	4x>21	2,4,4,5, >21	7x1,22x 2,3x3,4, 3x6,2x >21
median; A:D			>21 14:6	>21 4:0	>21 5:0	>21 3:2	>21 5:0	>21 4:1	>21 5:0	>21 4:0	4 2:3	>21 4:0	4 1:4	2 2:36

Pooled Data for Protection against A66. by various PspAs														
Exp.	CFU A66	MICE	Days to Death/immunogen											
			FL-R36A/ D39	Rx1 BC100	JD908 (WU2)	JS1020 (BG9739)	BG9739 bc100	EF5668	L81905 FL	L81905 bc100	JS5010.3 FL (DBL5)	DBL5 bc100	JS3020 (DBL6A)	control
P values			<0.0001 <0.0001	0.0002 0.0001	<0.0001 <0.0001	0.004 0.0075	0.0002 <0.0001	0.0006 0.006	<0.0001 <0.0001	0.0002 0.0001	0.0025 n.s.	0.0002 0.0001	0.015 n.s	
Mini Pools			R36A/Rx1/WG44.1		JD908	BG9739		EF5668	L81905		DBL5 3,4,4,4,6x>21		DBL6A	Control
			>21 18:6		>21 5:0	>21 8:2		>21 4:1	>21 9:0		>21 6:4		4 1:4	2 2:36
P values rank/a:d			<0.0001		<0.0001 <0.0001	<0.0001		0.0006 0.006	<0.0001		0.0004		0.015 n.s.	
Score % alive			+ + + 72 71		+ + + 100 100	+ + + 80 79		+ + + 75 74	+ + + 100 100		+ + + 60 58		+ ± 20 16	5 0
A66 challenge			R36A/Rx1/WG44.1		JD908	BG9739		EF5668	L81905		DBL5		DBL6A	

A66 challenge	days of death	median days alive	alive: dead	*P* - days to death	*P* - alive:dead	Score	% alive	% protected
All immune	2,2,4x3,7x4, 5,8,13,50x> 21	>21	50:16	<0.0001	<0.0001	+++	76	75
All controls	7x1,22x2,3x 3,4,3x6,2x> 21	2	2:36				5	0

TABLE 43

Pooled Data for Protection against EF10197. by various PspAs									
Exp.	CFU EF 10197	Mice	Days to Death/immunogen						
			Rx1 BC100	JS1020 (BG9739)	L81905	JS3020 (DBL6A)	EF5668	JS5010.3 FL (DBL5)0	control
E140	3.00	CBA/N	5x>21						2,2,2
MI BCG	2.70	CBA/N	*						2,2,2,2,2
E129	3.34	CBA/N		8,4x>23					2,2,2,2,9

* This was a passive protection study. Its controls have been included to increase the numbers of control mice.

TABLE 44

Pool of Pools for protection against EF10197					
	Group	Delay in death and/or survival		Survival	
line	Description	days to death (median)	*P* values etc.	alive:dead	*P* values etc.
1a	Rx1 (E140)	5x > 21	0.017 vs 1b 0.0013 vs 4b	5:0	0.018 vs 1b 0.0008 vs 4b
3a	JS1020 (E129)	8, 4x > 23	0.0007 vs 3b	4:1	0.024 vs 3b
4a	all immune	8, 9x > 21	< 0.0001 vs 4b	9:1	0.0002 vs 4b
1b	Rx1 controls (E140)	2,2,2		0:3	
2b	MI BCG	2,2,2,2,2		0:5	
3b	JS1020 cont. (E129)	2,2,2,2,9		0:5	
4b	all control (without MI BCG)	2,2,2,2,2,2,2,9		0:8	

TABLE 45

Summary of protection against EF10197							
Immunogen	alive:dead	% alive	% protected	median DOD	*P* time alive	*P* alive:Dead	Score*
Rx1	5:0	100	100	>21	0.017	0.018	+++
JS1020	4:1	80	80	>21	0.0007	0.024	+++
all immune	9:1	90	90	>21	<0.0001	0.0002	+++
all controls	0:8	0	0	2	–	–	–

TABLE 46

Pooled Data for Protection against ATCC6303. by various PspAs

Exp.	CFU ATCC 6303	Mice	Days to Death/immunogen						
			Rx1 BC100	JS1020 (BG9739)	L81905	JS3020	EF5668	JS5010.3FL (DBL5)0	control
E140	2.30	CBA/N	5 x >21						4,4x5
E129	3.80	CBA/N		n.v.					

TABLE 47

Pool of Pools for protection against ATCC6303

	Group	Delay in death and/or survival		Survival	
line	Description	days to death (median)	*P* values etc.	alive: dead	*P* values etc.
1a	Rx1(E140)	5x>21 (>21)	0.0040	5:0	0.0004
1b	Rx1 controls (E140)	4,4x5 5	–	0:5	–

TABLE 48

Summary of protection against ATCC6303

Immunogen	alive:dead	% alive	% protected	median DOD	*P* time alive	*P* alive: dead	Score*
Rx1	5:0	100	100	>21	0.004	0.004	+++
Rx1 controls	0:5	0	0	5	–	–	–

* +++ = statistically significant protection against death with $\geq$50% protected.

TABLE 49

Pooled Data for Protection against BG9739, by FL PspAs

Exp.	CFU BG9739	Mice	Days to Death/immunogen											
			R36A FL	BC100 (Rx1)	JD908 (WU2)	JS1020 (BG9739	bc100 (BG9739)	EF329 6 FL	EF566 8 FL	bc100 (L81905)	Js5010 .3 FL (DBL5)	bc100 (DBL5)	JS3020 (DBL6A)	control
E140	2.76	CBA/N		3,3,10,11										2,2,3
E104	2.89	Xid				6,6,7,8,8					2,2,2,3 ,4		2,2,2,2, 3	2,2,3,5,5
E125	3.56	CBA/N					5,5,5,7			4,5,13,> 21		2,2,2,4		3,3,4,4,5 ,6
E172	3.71	CBA/N			6,7,3 x >21									3,4,6,6,7
E124	3.76	Xid									2,2,2,2 ,3		2,2,2,2, 9	2,2,2,2,2
E084	4.05	BALB/c				4x2,2x>1 4								9x2
E144	4.09	Xid	2,3,6, >2,1					2,3,3,7, >10	2,3,3,3 ,4					2,2,2,3,3
All			2,3,3,6 >2,1	3,3,10,11	6,7,3 x >21	4X2,6,6,7 ,8,8,2X> 21	5,5,5,7	2,3,3,7, >21	2,3,3,3 ,4		7x2,3, 3,4		8x2,3,9	21x2,7x2 ,3x4,3x5, 3x6,7
median			3	3,10	>21	6	5	3	3	5,13	2	2	2	2
a:d			1:4	0:4	3:2	2:9	0:4	1:4	0:5	1:3	0:10	0:4	0:10	0:38
P rank														
P a:d														

TABLE 50

Pooled Data for Protection against BG9739, by bc100s and FL PspAs

Exp.	CFU BG9739	Mice	Days to Death/immunogen											
			R36A FL	BC100 (Rx1)	JD908 (WU2)	JS1020 (BG9739	bc100 (BG9739)	EF3296 FL	EF5668 FL	bc100 (L81905)	JS501 0.3 FL (DBL5)	bc100 (DBL5)	JS3020 (DBL6A)	control
E140	2.76	CBA/N		3,3,10,11										2,2,3
E104	2.89	Xid				6,6,7,8,8					2,2,2,3, 4		2,2,2,2, 3	2,2,3,5,5
E125	3.56	CBA/N					5,5,5,7			4,5,13, > 21		2,2,2,4		3,3,4,4,5,6
E172	3.71	CBA/N			6,7,3 x >21									3,4,6,6,7
E124	3.76	Xid									2,2,2,2, 3		2,2,2,2, 9	2,2,2,2,2
E084	4.05	BALB/c				4x2,2x>14								9x2
E144	4.09	Xid	2,3,6,>2,1					2,3,3,7, >10	2,3,3,3, 4					2,2,2,3,3

Pooled Data for Protection against BG9739, by bc100s and FL PspAs														
Exp.	CFU BG9739	Mice	Days to Death/immunogen											
			R36A FL	BC100 (Rx1)	JD908 (WU2)	JS1020 (BG9739	bc100 (BG9739)	EF3296 FL	EF5668 FL	bc100 (L81905)	JS501 0.3 FL (DBL5)	bc100 (DBL5)	JS3020 (DBL6A)	control
FL + bc100 BG9739			R36A/Rx1/D39		WU2	BG9739		EF3296	EF5668	L81905	DBL5		DBL6A	Cont.
All			2,4x3,6,10,11,>21		6,7,3x>21	4x2,3x5,2x6,2x7,2x8,2x>21		2,3,3,7,>21	2,3x3,4	4,5,13,>21	10x2,3,3,4,4		8x2,3,9	21x2,7x3,3x4,3x5,3x6,7
median days alive			3		>21	6		3	3	5,13	2		2	2
alive:dead			1:8		3:2	2:13		1:4	0:5	1:3	0:14		0:10	0:38
P - days alive			0.0096		<0.0001	0.0013		n.s.	n.s.	0.0022	n.s.		n.s.	
P - alive:dead			n.s.		0.0008	n.s		n.s.	n.s.	n.s.	n.s.		n.s.	
Score			+		+++	+		0+	0	0	0		0	
% alive			11		60	13		25	0	0	0		0	0
% protected			11		60	13		25	0	0	0		0	0
BG9739 challenge			R36A/Rx1/D39		WU2	BG9739		EF3296	EF5668	DBL5	DBL5		DBL6A	Cont.

BG9739	days of death	median days of death	alive:dead	*P* value based on days to death	*P* value based on alive:dead	Score	% Alive	%	
All immune		3	8.59	0.0009	0.023	++	12	12	
All controls		2	0:38						

TABLE 51

Pooled Data for Protection against EF3296. by various PspAs								
Exp.	CFU EF3296	Mice	Days to Death/immunogen					
			Rx1 BC100	JD908 WU2	JS1020 (BG9739)	JS5010.3FL (DBL5)	JS3020 (DBL6A)	control
E84[1]	3.99	BALB/c			4x2, >14			9x2
E140	2.92	CBA/N	3,4,6,>21					3,3,3
E104	3.11	CBA/N			4,5,5,5,6	2,2,2,3,3	2,2,3,4,5	2,2,2,3,4
E124	3.94	CBA/N				1,1,2,2,2	1,1,2,2,2	1,1,2,2,2
E172	4.06	CBA/N						3,4x6
All			3,4,6,>21	3,3,5,5,>21	4X2,4,3x5, 6,>21	1,1,5x2,3,3	1,1,5x2,3, 4,5	1,1,15x2, 5x3,4,4x6
median days to death			5	5	4.5	2	2	2
alive:dead			1:3	1:4	1:9	0:9	0:10	0:27
P-days to death			0.0077	0.0094	n.s.	n.s.	n.s.	
P-alive:dead			n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Score			+ ±	+ ±	0+	0	0	
% alive			25	20	10	0	0	0
% prot.								
Best								

EF3296 challenge	Rx1 BC100	JD908 WU2	JS1020 (BG9739)	JS5010.3FL (DBL5)	JS3020 (DBL6A)	control

EF3296 challenge	median days alive	alive : dead	*P* - days : to death	*P* - alive : dead	Score%	% alive	% prot
All immune	3	3:35	n.s.	n.s.	0	8	8
All control	2	0:27					

TABLE 52

Pooled Data For Protection against EF5668, by various FL-PspAs and bc100s

Exp.	CFU EF5668	Mice	Days to Death/immunogen									
			R36A	Rx1 BC100	JD908 (WU2)	JS1020(BG9 739)	EF3296	EF5668	L81905	JS5010.3 FL DBL5	JS3020 DBL6A	control
E143	3.0	CBA/N						5x>10				1,1,2,2, >10
E140	3.59	CBA/N		4,6, 12, >21								2,4,6
E171	3.69	CBA/N			2,2,2,3 >21				3,3,4, 2x>21			1,3,66,7
E124	3.90	CBA/N								3,3,3x >21	3,4,5,6,6	3,3,34,9
E145	3.94	CBA/N	3,4,416>19			2,10 3x>19	2,4, 13, 2x>19					2,3,34, >21
Pool			3,3x4,6,12,16 2x>21		2,2,2 3,<21	2,10, 3x>21	2,4, 13, 2x>21	5x<21	3,3,4 2x>21	3,3, 3x >21	3,4, 5,6	3x1,4x2 6x3,3x4 3x6,7,9, 2x>21
median days alive			6		2	<21	13	<21	4	<21	5	3
alive:dead			2:7		1:4	3:2	2:3	5:0	2:3	3:2	0:5	2:21
P - days alive			0.013		n.s.	0.0187	n.s.	0.001	n.s.	n.s.	n.s.	
P - alive:dead			n.s.		n.s.	0.027	n.s.	0.0002	n.s.	0.027	n.s.	
Score			+		0	+++	0+	+++	0+	+	0+	
% alive			22		25	60	40	100	40	60	0	9
% prot.			14		18	56	34	100	34	56	-10	9

EF5668	R36A/Rx1/D39	WU2	BG9739	EF3296	EF5668	L81905	DBL5	DBL6A	control

Summary of protection against EF6796

Immunogen	alive:dead	% alive	% protected	median DOD	*P* - time alive	*P* alive vs. dead
Rx1	4:0	100	100	>21	0.029	0.029
controls	0:3	0	0	1	--	--

TABLE 53

Pooled Data for Protection against DBL6A. by various FL PspAs and bc100 PspAs

Exp.	CFU	Mice	Days to Death/immunogen												
	DBL6A		BC100 Rx1	R36A	JD908 WU2	JS1020 BG 9739	bc100 BG9739	EF3296	EF5668	L81905 FL	bc100 L81905	JS 5010.3 DBL5	bc100 DBL5	JS2030 DBL6A	control
E171	2.69	CBA/N			6,7,8, 9,>21					3,3,7,9, >21					2,3,4,6,6
E152	3.24	CBA/N					15,3x>21				7,16, 2x>21		8,10, 13, 21		3x3,4,3x6
E140	3.25	CBA/N	4x>21												4,7,7
E146	3.57	CBA/N		7,8,10, 2x>21				6,8,9,10,10	10,13,3x>21			7,8,12, 13,13		9,4x>21	4,45,5,18
E129	4.14	CBA/N				3,6,8,10,13									4,5,6,8,>23
Total															

Name of Pools	R36A/Rx1/D39	WU2	BG9739	EF3296	EF5668	L81905	DBL5	DBL6A	controls
Pooled data	7,8,10,6x>21	6,8,9, >21	3,6,8,10,13,15,3x>21	6,8,9, 10,10	10,13,3x>21	3,3,7,7,9,16,2x>21	7,8,8,10,12, 3x13,21	9,4x>21	2,4x3, 6x4, 3x5, 6x6,7,7,8,18, >21
median days alive	>21	8.5	13	9	>21	8	12	>21	5
alive:dead	6:3	1:3	3:6	0:5	3:2	2:6	0:9	4:1	1:24
P - days alive	<0.0001	0.0082	0.0025	0.0036	0.0001	0.037	0.002	<0.0001	
P - alive:dead	0.0019	n.s.	0.048	n.s.	0.0093	n.s.	n.s.	0.0009	
Score	+++	+±	++	+±	+++	+±	+±	+++	

	7	25	33	0	60	25	0	80	4
	66	22	30	-4	58	22	-4	79	0
DBL6A	R36A/Rx1/D39	WU2	BG9739	EF3296	EF5668	L81905	DBL5	DBL6A	controls

DBL6A	days of death	median days of death	alive:dead	*P* value based on days to death	*P* value based on alive:dead	Score	% alive	% protected
All immune		12.5	19:35	<0.0001	0.0019	++	35	33
All control		5	1:24					

TABLE 54

Pooled Data for Protection against BG9163 by various PspAs

Exp.	CFU BG9163	Mice	Days to Death/immunogen				
			Rx1	Rx1.BCG	JS1020 (BG9739	all immune	control
E169	2.67	CBA/N	5x>24				4,5,8,8,>24
E140	3.14	CBA/N	n.v.				
E129	4.0	CBA/N			12,4x>23		7,9,9,13,>23
E028	6.217	CBA/N		6,3x>21			5,6,8,10
Immunogens			Rx1/R36A/D39		BG9739	all immune	control
Pooled Data			6,8x>21		12,4x>21	6,12,12x>21	4,5,7,8,8,9,9,12, 2x>21
median days alive			>21		>21	>21	8.5
alive:dead			8:1		4:1	12:2	2:8
P - days alive			0.0086		0.0097	0.0027	
P - alive:dead			0.0045		0.047		0.0022
% alive			89		80	86	20
% port.			86		75	83	0
score			+++		+++	+++	
BG9163 Challenge			Rx1/R36A/D39		BG9739	all immune	control

EF5668	days of death	median days of death	alive:dead	*P* value based on days to death	*P* value based on alive:dead	Score	% alive	% protected
All immune		8	18:26	0.0015	0.005	++	41	35
All control		3	2:21					

TABLE 55

Pooled Data for Protection against L81905, by various FL-PspAs

Exp.	CFU L81905	Mice	Days to Death/immunogen											
			R36A	BC100 (Rx1)	JD908 (WU2)	JS1020 (BG 9739)	bc100	EF3296	EF5668	bc100 L81905	JS 5010.3 (DBL5)	bc100 (DBL5)	JS3020 (DBL6A)	control
E172	2.45	CBA/N			3,4,5,6, 6									3,3,4,4,4
E140	3.11	CBA/N		2,5,5,6, 8										2,2,2,3,3
E084	3.86	BALB				2,2,5x> 14								1,8x2
E104	-3.5	CBA/N				3,7,8,8, 11					3,3,3,2x >22		3,4,5,5, 6	2,4,4,4,5
E124	-3.5	CBA/N									2,2,2,2, 3		2,2,2,3, 5	1,2,2,2,2
E125	3.6	CBA/N					5,6,8,8			3,4,6,8		4,5,5,5		2,2,3,5,5,5
E144	4.11	CBA/N	3,3,5,6, >10					6,6,6,2x >10	2,2,3,3, 3					2,2,3x3
All			3,3,5,6, >21		3,4,5,6, 6	2,2,3,7, 8,8,11,5 x>21	5,6,8,8	6,6,6,2x >10	2,2,3,3, 3	3,4,6,8	4x2,4x3, 2x>21	4,5,5,5	3x2,3,3, 4,3x5,6	1,1,20x2,8x3,6x4,4x 5
median			5	5	5	>21	7	6	3	5	3	5	3.5	2
alive: dead			1:4	0:5	0:5	5:7	0:4	2:3	0:5	0:4	2:8	0:4	0:10	0:40
P rank														
P a:d														
score														

TABLE 56

Protection against L81905, by various bc100s & FL-PspAs pooled together

Exp.	CFU L81905	Mice	Days to Death/immunogen											
			R36A	BC100 (Rx1)	JD908 (WU2)	JS1020 (BG 9739)	bc100	EF3296	EF5668	bc100 L81905	JS 5010.3 (DBL5)	bc100 (DBL5)	JS3020 (DBL6A)	control
E172	2.45	CBA/N			3,4,5,6, 6									3,3,4,4,4
E140	3.11	CBA/N		2,5,5,6, 8										2,2,2,3,3
E084	3.86	BALB				2,2,5x> 14								1,8x2
E104	-3.5	CBA/N				3,7,8,8, 11					3,3,3,2x >22		3,4,5,5, 6	2,4,4,4,5
E124	-3.5	CBA/N									2,2,2,2, 3		2,2,2,3, 5	1,2,2,2,2
E125	3.6	CBA/N					5,6,8,8			3,4,6,8		4,5,6,5		2,2,3,5,5,5
E144	4.11	CBA/N	3,3,5,6, >10					6,6,6,2x >10	2,2,3,3, 3					2,2,3x3
Pooled			2,3,3,3x5,6,6,8,>21		3,4,5,6, 6	2,2,3,5,6,7,4x8,11, 5x>21		6,6,6,2x >10	2,2,3,3, 3	3,4,6,8	4x2,4x3,4,5,5,5, 2x>21		3x2,3,3, 4,3x5,6	1,1,20x2 8x3.6x4, 4x5
median days alive			5		5	8		6	3	5	3		3.5	2
alive:dead			1:9		0:5	5:11		2:3	0:5	0:4	2:12		0:10	0:40
P - days alive			0.0005		0.0035	<0.0001		0.0002	n.s..	0.01	0.035		0.044	
P alive:dead			n.s.		n.s.	0.0001		0.01	n.s.	n.s.	n.s.		n.s.	
score			+		+	++		++	0	+	+		+	
% alive			10		0	31		40	0	0	14		0	0
% protected														
challenge with L81905			R36A/Rx1/D39		WU2	BG9739		EF3296	EF5668	L81905	DBL5		DBL6A	controls

L81905 challenge	days of death	median days of death	alive:dead	*P* value based on days to death	*P* value based on alive:dead	Score	% alive	% protected
All immune		5	10:59	<0.0001	0.008	++	14	14
All control		2	0:40					

TABLE 57

Pooled Data for Protection against DBL5 by various FL-PspAs & bc100s

Exp.	CFU DBL5	Mice	Days to Death/immunogen									
			R36A	BC100 Rx1	JS1020 BG9739	bc100 JS1020	EF5668	bc100 L81905	JS5010.3 DBL5	bc100 DBL5	JS3020 DBL6A	control
E84[1]	3.90	BALB/c			6x2							9x2
E140	3.27	CBA/N		4,4,5,5, 5								2,2,2
E104	3.39	Xid			3,3,6, >22,>22				7,7,15, >22,>22		2,2,4,5, 5	2,4x3
E124	3.76	Xid							2,2,2,5,> 15		5x2	1,1,2,2,2
E125	3.81	CBA/N				3,3,4,5		3,3,4,4		2,2,2,> 21		5x2,5
E144	4.13	XID	3,3,3,3, >10				2,2,3,4, 4					5x2
total												
Name of Pool			R36A/Rx1/D39		BG9739		EF5668	L81905	DBL5		DBL6A	controls
Pooled Data			4x3,2x4,3x5,>21		6x2,4x3,4,5, >21,>21		2,2,3,4, 4	3,3,4,4	6x2,5,7,7,15,4x>21		7x2,4,5, 5	1,1,26x2,4x3,5
median days alive			4		3		3	3.5	6		2	2
alive:dead			1:9		2:12		0:4	0:4	4:10		0:10	0:32
P- days alive			<0.0001		0.0063		.041	0.001	0.0025		n.s.	
P alive:dead			n.s.		n.s.		n.s.	n.s.	0.0056		n.s.	
score			+		+		+*	+	++		0	
% alive			10		14		0	0	29		0	0
% protected			10		14		0	0	29		0	0
DBL5 challenge			R36A/Rx1/D39		BG9739		EF5668	L81905	DBL5		DBL6A	controls

[1] This immunization was with cell eluted PspA. Note BALB/cJ mice were used. Also note 10^4 Challenge CFU.

DBL5 challenge	days of death	median days of death	alive:dead	*P* value based on days to death	*P* value based on alive:dead	Score	% alive	% protected
All immune		3.5	7:49	0.0001	0.034	++	3.6	3.6
All control		2	0:33					

TABLE 58

Pooled Data for Protection against EF6796 by various PSpAs									
Exp.	CFU WU2	Mice	Days to Death/immunogen						
			Rx1 BC100	JS1020 (BG9739)	L81905	JS3020 (DBL6A)	JS5010.3FL	DBL5 bc100	control
E140	3.75	CBA/N	4x >21						1,1,1
E28	?	BALB	n.v.						

TABLE 59

Pool of Pools for protection against EF6796						
line	Group Description	Delay in time to death and/or survival			Protection against death	
		days to death (median DOD)		*P* values etc.	alive:dead	*P* values etc.
1a	Rx1	4x >21	(<21)	0.029	4:0	0.029
1b	Rx1 controls	1,1,1	(1)	--	0:3	--

TABLE 60

Pooled Data for Protection against BG7322. by various FL-PspAs & bc100s

Exp.	CFU BG 7322	Mice	Days to Death/immunogen										
			D39/ R36A	Rx1 BC100	JD908 (WU2)	bc100 BG9739	EF3296	EF5668	bv100 L81905	JS5010.3 DBL5	bc100 DBL5	JS3020 DBL6A	control
E171	2.78	CBA/N			10,15,3x>21								1,3,6,6,7
S143	3.0	CBA/N						7,8x>10					2,2,3,4,7,7,8,8
E140 BC100	3.14	CBA/N		4x>21									3,6,6,>21
E152	3.11	CBA/N				12,13,16,>21			10,12,13,>21		>21, >21, >21, >21		6,7,7,8,8,9,14
E146	3.57	CBA/N	18,20,3x>21				5,3xx6, 10			6,10,11, 11,19		4,8,11,18,>21	4,5,5,6,>21
E169	3.94	CBA/N	5x>21										2,5,5,6,7
Immunogens			R36A/Rx1/D39		JD908	BG9739	EF3296	EF5668	L81905	DBL5		DBL6A	Cont.
Pools			18,20,12x>21		10,15,3x>21	12,13,16,>21	5,3,x6, 10	7,8x>21	10,12, 13,>21	6,10,11,11,19>21, >21,>21,>21		4,8,11,18,>21	1,3x2,3,3, 4,4,5x5,7,x 6,6x7,4x8, 9,14,2x>21
median days alive			>21		>21	14.5	6	>21	12.5	>21		11	6
alive:dead			9:0		3:2	1:3	0:5	8:1	1:3	4:5		1:3	2:32
P - days alive			<0.0001		0.0007	0.001	n.s.	<0.0001	0.0013	0.0002		0.028	
P alive:dead			<0.0001		0.004	n.s.	n.s.	<0.0001	n.s.	0.0076		n.s.	
% alive			100		60	25	0	89	25	80		25	6
% protected			100		57	22	0	88	22	79		22	6
Score			+++		+++	+±	0	+++	+±	+++		+±	

BG7322 Challenge	R36A/Rx1/D39	JD908	BG9739	EF3296	EF5668	L81905	DBL5	DBL6A	Cont.

BG7322 Challenge	median days of death	alive:dead	*P* value based on days to death	*P* value based on alive:dead	Score	% alive	% protected
All immune	>21	30:25	<0.0001	<0.0001	+++	55	52
All controls	6	2:32					

EXAMPLE 8 - ABILITY OF PspA IMMUNOGENS TO PROTECT AGAINST INDIVIDUAL CHALLENGE STRAINS

In Example 7 some of the capsular type 2, 4, and 5 strains were not completely protected from death by immunization. In these studies the BALB/cByJ mouse was used instead of the hypersusceptible, immunodeficient CBA/N mouse used for the Example 7 studies. With the BALB/cJ mouse it was observed that immunization with PspA was in fact able to protect against death with capsular type 2, 4, and 5 pneumococci. This result is shown in the table below.

The data from Table 60A also demonstrates that a mixture of 4 - 5 full length PspAs was as effective, or more effective than immunization with a single PspA.

Table 60A. Days of death of BALB/cByJ mice after immunization with monovalent and polyvalent vaccine.

Challenge Strains					Immunogen			
strain	caps	PspA	pspA	Log	Days to Death			
name	type	type	B region clade	Challenge dose	1 mg R36A + CFA	4 - 5 valent mixture (0.5μg each). + CFA	JY2141 + CFA	None
D39	2	25	2	4.76	3, 4x >21	3, 4x >21	3, 4, 5, 11, >21	3, 3, 4, 4, 8
WU2	3	1	2	4.8	4x >21	4x >21	6, 3x >21	3, 4, 2x >21
A66	3	13	?	4.7	3, 3, >21, >21	2, 3x >21	2, 2, 3, 4	2, 3, 4, 4
BG9739	4	26	1	4.07 - 4.4	7, 8x >21	3, 8x >21	1, 5, 6, 6, 9, 4x >21	3, 3, 3, 4, 6, 7, 7, 2x >21
L81905	4	23	1	6.90 - 6.96	2, 2, 2, 2, 5, 5, 4x >21	2, 6, 8, 9, 6x >21	1, 1, 1, 1, 2, 3, 4, 5, 2x >21	1, 4x 2, 3x 3, 4, >21

EF5668	4	12	4	6.10-6.93	3, 3, 4, 7x >21	3x 3, 6x >21	4x 3, 4, 4, 6, 6, >21	3, 5x 4, 6, >21
DBL5	4	33	2	3.30	7, 14, 3x >21	3, 5, 5, 2x >21	2, 2, 2, 4, 6	4, 5, 5, 6, 9
DBL6A	6A	19	1	4.34	6, 9, 10, 11, >21	10,11,12,13, >21	3, 11, 11, 13, 16	8, 9, 11, 21, >21
BG7322	6B	21	?	3.9	8, 8, 3x >21	5x >21	6, 6, 7, 8, 10	2, 5, 6, 8, 8

• Note, numbers such as 8x >21 indicate that 8 mice lived greater than 21 days.

Note, JY2141 is a preparation from a strain that lacks PspA. None = no immunization.

Note, mice were given two immunizations with PspA two weeks apart and challenged intravenously 2 weeks after the last immunization. The first immunization was given with complete Freund's adjuvant (CFA) subcutaneously, the second immunization was given intraperitoneally in saline.

[1] 4 valent vaccine mixture R36A, BG9739, EF5668, and DBL5 -- all E180

[2] 4 valent vaccine mixture R36A, BG9739, DBL5, EF3296, D39 and DBL6A

[3] 5 valent vaccine mixture R36A, BG9739, DBL5, EF3296, EF5668

EXAMPLE 9 - CHARACTERIZATION OF PspA EPITOPES WITHIN PNEUMOCOCCAL STRAINS MC25-28

The strains examined came from a group of 13 capsular serotype 6B strains which have been identified that are members of a multiresistant clone, having resistance to penicillin, chloramphenicol, tetracycline, and some have acquired resistance to erythromycin. The pneumococcal isolates described in the following studies (MC25-28) are members of this 6B clone. Although previously thought to be geographically restricted to Spain (unlike the widespread multiresistant Spanish serotype 23F clone), members of this clone have been shown to be responsible for an increase in resistance to penicillin in Iceland (Soares, S., et al., J. Infect. Dis. 1993; 168: 158-163).

The following techniques were used to characterize the location of difference PspA epitopes:

Bacterial cell culture

Bacteria were grown in Todd-Hewitt broth with 0.5% yeast extract or on blood agar plates overnight at 37°C in a candle jar. Capsular serotype was confirmed by cell agglutination using Danish antisera (Statens Seruminstitut, Copenhagen, Denmark). The isolates were subtyped as 6B by Quellung reaction, utilizing rabbit antisera against 6A or 6B capsule antigen.

Bacterial lysates

Cell lysates were prepared by incubating the bacterial cell pellet with 0.1% sodium deoxycholate, 0.01% sodium dodecylsufate (SDS), and 0.15 M sodium citrate, and then diluting the lysate in 0.5M Tris hydrochloride (pH 6.8). Total pneumococcal protein in the lysates was quantitated by the bicinchoninic acid method (BCA Protein Assay Reagent; Pierce Chemical Company, Rockford, IL).

PspA serotyping

Pneumococcal cell lysates were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and developed as Western blots using a panel of seven MAbs to

PspA. PspA serotypes were assigned based on the particular combination of MAbs with which each PspA was reactive.

Colony immunoblotting

A ten mL tube of Todd-Hewitt broth with 0.5% yeast extract was inoculated with overnight growth of MC25 from a blood agar plate. The isolate was allowed to grow to a concentration of 10^7 cells/mL as determined by an O.D. of 0.07 at 590nm. MC25 was serially diluted and spread-plated on blood agar plates to give approximately 100 cells per plate. The plates were allowed to grow overnight in a candle jar, and a single blood agar plate with well-defined colonies was selected. Four nitrocellulose membranes were consecutively placed on the plate. Each membrane was lightly weighted and left in place for 5 min. In order to investigate the possibility of phase-variation between the two proteins detected on Western blots a single colony was picked from the plate, resuspended in ringer's solution, and spreadplated onto a blood agar plate. The membranes were developed as Western blots according to PspA serotyping methods.

When the strains MC25-28 were examined with the panel of seven MAbs specific for different PspA epitopes, all four demonstrated the same patterns of reactivity (Fig. 14). The MAbs XiR278 and 2A4 detected a PspA molecule with an apparent molecular weight of 190 kDa in each isolate. In accordance with the PspA serotyping system, the 190 kDa molecule was designated as PspA type 6 because of its reactivity with XiR278 and 2A4, but none of the five other MAbs in the typing system. Each isolate also produced a second PspA molecule with an apparent molecular weight of 82 kDa. The 82 kDa PspA of each isolate was detected only with the MAb 7D2 and was designated as type 34. No reactivity was detected with MAbs Xi126, Xi64, 1A4, or SR4Wr. Results from the colony immunoblotting showed that both PspAs were present simultaneously in these isolates under *in vitro* growth conditions. All colonies on the plate, as well as all of the progeny form a single colony, reacted with MAbs XiR278, 2A4, and 7D2.

EXAMPLE 10 - SOUTHERN BLOT ANALYSIS OF CHROMOSOMAL DNA ISOLATED FROM PNEUMOCOCCAL STRAINS MC25-28

Pneumococcal chromosomal DNA was prepared by the Youderian method (Sheffield, J.S., et al., Biotechniques, 1992; 12: 836-839). Briefly, for a 500 ml culture in THY or THY with 1% choline, cells were centrifuged at 8000 rpm in GSA rotor for 30 minutes at 4°C. The supernatant was decanted, and the cells were washed with 1 to 2 volumes of sterile water to remove choline, if used. This step was only necessary when sodium deoxycholate was used. The wasted cells were centrifued twice a 8000 rpm in GSA rotor for 10 minutes. Cells were resuspended in 3.5 ml TE buffer, containing 1% SDS or 1% sodium deoxycholate, and incubated at 37°C for 15 minutes if sodium deoxycholate was used. If SDS was used, incubation at 37°C was not necessary. The cells were incubated at 65°C for 15 minutes, and 1/5 volume of 5.0 M potassium acetate was added, and the cell suspension was incubated for 30 minutes at 65°C.

The cells were placed on ice for 60 minutes, and centrifuged at 12,000 rpm in an SS-34 rotor for 10 minutes. The supernatant was transferred to a clean centrifuge tube, and 2 volumes of cold 95% ethanol was added. After mixing, DNA was spooled on to a glass pasteur pipet, and air dried. The DNA was resuspended in 4 ml TE, and 4.0 g cesium chloride was added. The solution was split into two aliquots in ultracentrifuge tubes, and the tubes were filled to their maximum capacity using 1.0 g/ml cesium chloride in TE. Before closing the tubes, 300 ml of 10 ug/ml ethidium bromide was added.

The solution was centrifuged at 45,000 rpm overnight, or for 6 hours at 55,000 rpm. The chromosomal band was extracted using a gradient, at least 6 times with 1 volume each salt-saturated isopropanol. The aqueous phase was extracted by adding 2 volumes 95% ethanol. The DNA came out of solution immediately, and it was spooled on to a pasteur pipet. The DNA pellet was washed by dipping the spooled DNA in 5 ml 70% ethanol. The DNA was air dried, and resuspended in the desired volume of TE, e.g., 500 ul.

The cells were harvested, washed, lysed, and digested with 0.5% (st/vol) SDS and 100μg/mL proteinase K at 37°C for 1 h. The cell wall debris, proteins, and polysaccharides were complexed with 1% hexadecyl trimethyl ammonium bromide (CTAB) and 0.7M sodium chloride at 65°C for 20 min., and then extracted with chloroform/isoamyl alcohol. DNA was precipitated with 0.6 volumes isopropanol, washed, and resuspended in 10mM Tris-HCl, 1mM EDTA, pH 8.0. DNA concentration was determined by spectrophotometric analysis at 260 nm (Meade, H.M. et al., J. Bacteriol 1982; 149: 114-122; Silhavy, T.J. et al., Experiments with Gene Fusion, Cold Spring Harbor: Cold Spring Harbor Laboratory, 1984; and Murray, M.G., et al., Nucleic Acids Res. 1980; 8 4321-4325).

Probe preparation

5′ and 3′ oligonucleotide primers homologous with nucleotides to 26 and 1967 to 1990 of Rx1 *psp*A (LSM13 and LSM2, respectively) were used to amplify the full length *psp*A and construct probe LSM*psp*A13/2 from Rx1 genomic DNA. 5′ and 3′ oligonucleotide primers homologous to nucleotides 161 to 187 and nucleotides 1093 to 1117 (LSM12 and LSM6, respectively) were used to amplify the variable α-helical region to construct probe LSM*psp*A12/6. PCR generated DNA was purified by Gene Clean (Bio101 Inc., Vista, CA) and random prime-labeled with digoxigenin-11-dUTP using the Genius 1 Nonradioactive DNA Labeling and Detection Kit as described by the manufacturer (Boehringer Mannheim, Indianapolis, IN).

DNA electrophoresis

For Southern blot analysis, approximately 10μg of chromosomal DNA was digested to completion with a single restriction endonuclease (Hind III, Kpn 1, EcoRI, Dra I, or Pst I), then electrophoresed on a 0.7% agarose gel for 16-48 h at 35 volts. For PCR analysis, 5μL of product were incubated with a single restriction endonuclease (Bcl 1, BamH I, Bst I, Pst I, Sac I, EcoR I, Sma I, and Kpn I), then electrophoresed on a 1.3% agarose gel for 2-3 h at 90 volts. In both cases, 1 kb DNA ladder was used for molecular weight markers (BRL, Gaithersburg, MD), and gels were stained with ethidium bromide for 10 min and photographed with a ruler.

Southern blot hybridization

The DNA in the gel was depurinated in 0.25N HCl for 10 min, denatured in 0.5M NaOH and 1.5M NaCl for 30 min, and neutralized in 0.5M Tris-HCl (pH 7.2), 1.5M NaCl and 1mM disodium EDTA for 30 min. DNA was transferred to a nylon membrane (Micron Separations INC, MA) using a POSIBLOT pressure blotter (Stratagene, LaJolla, CA) for 45 min and fixed by UV irradiation. The membranes were prehybridized for 3 h at 42°C in 50% formamide, 5X SSC, 5X Denhardt solution, 25mM sodium phosphate (pH 6.5), 0.5% SDS, 3% (wt/vol) dextran sulfate and 500μg/mL of denatured salmon sperm DNA. The membranes were then hybridized at 42°C for 18 h in a solution containing 45% formamide, 5X SSC, 1X Denhardt solution, 20mM sodium phosphate (pH 6.5), 0.5% SDS, 3% dextran sulfate, 250μg/mL denatured sheared salmon sperm DNA, and about 20ng of heat-denatured digoxigenin-labeled probe DNA. After hybridization, the membranes were washed twice in 0.1% SDS and 2X SSC for 3 min at room temperature. The membranes were washed twice to a final stringency of 0.1% SDS in 0.3X SSC at 65°C for 15 min. This procedure yielded a stringency greater than 95 percent. The membranes were developed using the Genius 1 Nonradioactive DNA Labeling and Detection Kit as described by the manufacturer (Boehringer Mannheim, Indianapolis, IN). To perform additional hybridization with other probes, the membranes were stripped in 0.2N NaOH/0.1% SDS at 40°C for 30 min and then washed twice in 2X SSC.

PCR

5′ and 3′ primers homologous with the DNA encoding the N- and C-terminal ends of PspA (LSM13 and LSM2, respectively) were used. Reactions were conducted in 50μL volumes containing 0.2mM of each dNTP, and 1μL of each primer at a working concentration of 50mM. $MgCl_2$ was used at an optimal concentration of 1.75mM with 0.25 units of Taq DNA polymerase. Ten to thirty ng of genomic DNA was added to each reaction tube. The amplification reactions were performed in a thermal cycler (M.J. Research, Inc.) using the following three step program: Step 1 consisted of a denaturing temperature of 94°C for 2 min; Step 2 consisted of 9 complete cycles of a denaturing temperature of 94°C for 1 min, an annealing temperature of 50°C for 2 min, and an extension temperature of 72°C for 3 min; Step 3 cycled for 19 times with a denaturing temperature 94°C for 1 min, an annealing temperature of 60°C for 2 min, and an extension temperature of 72°C for 3 min; and at the end of the last cycle, the samples were held at 72°C for 5 min to ensure complete extension.

Band size estimation

Fragment sizes in the molecular weight standard and in the Southern blot hybridization patterns were calculated from migration distances. The standard molecular sizes were fitted to a logarithmic regression model using Cricket Graph (Cricket Software, Malvern, PA). The molecular weights of the detected bands were estimated by entering the logarithmic line equation obtained by Cricket Graph into Microsoft Excel (Microsoft Corporation, Redmond, WA) in order to calculate molecular weights based on migration distances observed in the Southern blot.

Since most strains contain a *psp*A gene and a *pspC* gene, it was expected that if an extra gene were present one might observe at least three *psp*A homologous loci in isolates MC25-28. In Hind III digests of MC25-28 each strain revealed 7.7 and 3.6 kb bands when probed with LSM*psp*A13/2 (Figure 15A and 15C). In comparison, when Rx1 DNA was digested with Hind III and hybridized with LSM*psp*A13/2, homologous sequences were detected on 9.1 and 4.2 kb fragments, as expected from previous studies with PspA (Figure 15A). Results consistent with two *psp*A-homologous genes in MC25-28 were obtained with two *psp*A-homologous genes in MC25-28 digested using four additional enzymes (Table 61).

Table 61. Chromosomal RFLPs with probe LSM*psp*A13/2 for isolates MC25-28 and Rx1

Restriction Enzyme	Strains Examined					Restriction Fragments (sizes in kilobases)	
	MC25	MC26	MC27	MC28	RX1	MC25-28	Rx1
Hind III	+	+	+	+	+	7.7, 3.6	9.1, 4.2
Kpn I	+	+	+	+	+	11.6, 10.6	10.6, 9.8
EcoR I	+				+	8.4, 7.6	7.8, 6.6
Dra I	+				+	2.1, 1.1	1.9, 0.9
Pst I	+				+	>14, 6.1	10.0, 4.0

The four isolates examined are all members of a single clone of capsular type 6B pneumococci isolated from Spain. These four isolates are the first in which two PspAs have been observed, i.e., PspA and PspC, based on the observation that bands of different molecular weights were detected by different MAbs to PspA. Mutation and immunochemistry studies have demonstrated that all of the different sized PspA bands from Rx1 are made of a single gene capable of encoding a 69kDa protein, supporting the assertion that two PspAs have been observed, i.e., PspA and PspC.

It has been observed that probes for the 5′ half of *pspA* (encoding the α-helical half of the protein) bind the *pspC* sequence of most strains only at a stringency of around 90%. With chromosomal digests of MC25-28, it was observed that the 5′ Rx1 probe LSM*pspA*12/6 (Figure 15D) bound two *pspA* homologous bands at even higher stringency. The same probe bound only the *pspA* containing fragment of Rx1 at the higher stringency (Figure 15B).

Further characterization of the *pspA* gene was done by RFLP analysis of PCR amplified *pspA* from each strain. Since previous studies indicated that individual strains yielded only one product, and since the amplification was conducted with primers based on a known *pspA* sequence, it was assumed that the product amplified from each strain represented the *pspA* rather than the *pspC* gene. When MC25-28 were subjected to this procedure, an amplified *pspA* product of 2.1 kb was obtained from each of the four strains. When digested with Hha I, this fragment yielded bands of 1.1, 0.46, 0.21 and 0.19 kb for each of the four isolates. A single isolate, MC25, was analyzed with eight additional enzymes. Using each restriction enzyme, the sum of the fragments was always approximately equal to the size of whole *pspA* (Figure 16). These results suggested that the 2.1 kb amplified DNA represents the amplified product of only a single *pspA* gene. Rx1 produced an amplified product of 2.0 kb and five fragments of 0.76. 0.468, 0.390, 0.349 and 0.120 kb when digested with Hha I as expected from its known *pspA* sequence.

There are several possible explanations for the observation of PspA and PspC in these strains but not in other strains. All isolates might make PspA and PspC in culture, but MAbs generally recognize only PspA (perhaps, in this isolate there has been a recombination between *pspC* DNA and the *pspC* locus, allowing that locus to make a product detected by MAb to PspA). All isolates can have PspA and PspC, but the expression of one of them generally does not occur under *in vitro* growth conditions. The *pspC* locus is normally a nonfunctional pseudogene sequence that, for an unexplained reason, has become functional in these isolates. Results from the colony immunoblotting of these isolates failed to show a detectable *in vitro* phase shift between either PspA type 6 (XIR278 and 2A4) or PspA type 34 (7D2) protein. This strengthens the second explanation, and suggests that the second PspA is these isolates is due to the *pspC* gene not being turned off during *in vitro* growth conditions.

Presumably, in these four strains, the second PspA protein is provided by the *pspC* DNA sequence. At high stringency, the probe comprising the coding region of the α-helical half of PspA recognized both pspA homologous sequences of MC25-18, but not the *pspC* sequence of Rx1. The finding indicated that the *pspC* sequence of MC25-28 is more similar to the Rx1 *pspA* sequence than the Rx1 *pspC* sequence. If the *pspC* sequence of these strains is more similar to *pspA* than most *pspC* sequences, it could explain why the products of *pspC* genes cannot generally be identified by MAbs.

EXAMPLE 11 - IDENTIFICATION OF CONSERVED AND VARIABLE REGIONS OF *pspA* AND *pspC* SEQUENCES OF *S. PNEUMONIAE*

The *S. pneumoniae* strains used in this study are listed in Table 62. The strains are human clinical isolates representing 12 capsular and 9 PspA serotypes. All strains were grown at 37°C in 100ml of Todd-Hewitt broth supplemented with 0.5% yeast extract to an approximate density of 5 x 10^8 cells/ml. After harvesting of the cells be centrifugation (2900 g, 10min), the DNA was isolated, and stored at 4°C in TE (10 mM Tris, 1mM EDTA, pH8.0).

Table 62. *Streptococcus pneumoniae* strains used.

Strain	Relevant phenotype	Reference
WU2	Capsular type 3, PspA type 1	Briles et al., 1981
D39	Capsular type 2, PspA type 25	Avery et al., 1944
R36A	Nonencapsuated mutant of D39, pspA type 25	Avery et al., 1944
Rx1	Derivatitve of R36A, PspA type 25	Shoemaker and Guild, 1974
DBL5	Capsular type 5, PspA type 33	Yother et al., 1986
DBL6A	Capsular type 6A, PspA type 19	Yother et al., 1986
A66	Capsular type 3, PspA type 13	Avery et al., 1944
AC94	Capsular type 9L, PspA type 0	Waltman et al., 1992
AC17	Capsular type 9L, PspA type 0	Waltman et al., 1992
AC40	Capsular type 9L, PspA type 0	Waltman et al., 1992
AC107	Capsular type 9V, PspA type 0	Waltman et al., 1992
AC100	Capsular type 9V, PspA type 0	Waltman et al., 1992
AC140	Capsular type 9N, PspA type 18	Waltman et al., 1992
D109-1B	Capsular type 23, PspA type 12	McDaniel et al., 1992
BG9709	Capsular type 9, PspA type 0	McDaniel et al., 1992
L81905	Capsular type 4, PspA type 25	McDaniel et al., 1992
L82233	Capsular type 14, PspA type 0	McDaniel et al., 1992
L82006	Capsular type 1, PspA type 0	McDaniel et al., 1992

Approximately 5μg of chromosomal DNA was digested with *Hind*III according to the manufacturer's instructions (Promega, Inc., Madison, WI). The digested DNA was subjected to electrophoresis at 35 mV overnight in 0.8% agarose gels and then vacuum-blotted onto Nytran® membranes (Schleicher & Schuell, Keene, NH).

The oligonucleotides uses were based on the previously determined sequence of Rx1 *psp*A. Their position and orientation relative to the structural domains of Rx1 pspA are shown in Figure 17. Labeling of oligonucleotides and detection of probe-target hybrids were both performed with the Genius System® according to manufacturer's instructions (Boehringer-Mannhein, Indianapolis, IN). All hybridizations were done for 18 hours at 42°C without formamide. By assuming that 1% base-pair mismatching results in a 1°C decrease in T_m arbitrary designations of "high" and "low" stringency were defined by salt concentration and temperature of post-hybridization washes. Homology between probe and target sequences was derived using calculated T_m by established methods. High stringency is defined as $\geq$ 90%, and low stringency is $\leq$ 85% base-pair matching.

PCR primers, which were also used as oligonucleotide probes in Southern blotting and hybridizations, were designed based on the sequence of *psp*A from pneumococcal strain Rx1. These oligonucleotides were synthesized by Oligos, Etc. (Wilson, OR), and are listed in Table 63.

Table 63. Oligonucleotide sequences.

Primer	5′ -> 3′
LSM111	CCGGATCCAGCTCCTGCACCAAAAC
LSM2	GCGCGTCGACGCTTAAACCCATTCACCATTGG
LSM3	CCGGATCCTGAGCCAGAGCAGTTGGCTG
LSM4	CCGGATCCGCTCAAAGAGATTGATGAGTCTG
LSM5	GCGGATCCCGTAGCCAGTCAGTCTAAAGCTG
LSM6	CTGAGTCGACTGGAGTTTCTGGAGCTGGAGC
LSM7	CCGGATCCAGCTCCAGCTCCAGAAACTCCAG
LSM9	GTTTTTGGTGCAGGAGCTGG
LSM10	GCTATGGCTACAGGTTG
LSM12	CCGGATCCAGCGTGCCTATCTTAGGGGCTGGT
LSM112	GCGGATCCTTGACCAATARRRACGGAGGAGGC

PCR was done with an MJ Research, Inc., Programmable Thermal Cycler (Watertown, MA), using approximately 10 ng of genomic pneumococcal DNA as template with designated 5′ and 3′ primer pairs. The sample was brought to a total volume of 50 µl containing a final concentration of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5mM $MgCl_2$, 0.01% gelatin, 0.5 µM of each primer, 200 µM of each deoxynucleoside triphosphate, and 2.5 U of *Taq* DNA polymerase. The samples were denatured at 94°C for 2 minutes and subjected to 10 cycles consisting of: 1 min at 94°C, 2 min at 50°C, and 3 min at 72°C, followed by 20 cycles of: 1 min at 94°C, 2 min at 60°C, and 3 min at 72°C. After 30 total cycles, the samples were held at 72°C for an additional 5 min prior to cooling to 4°C. The amplicons were then analyzed by agarose gel electrophoresis.

Oligonucleosides were used to probe *Hind*III digests of DNA from 18 strains of *S. pneumoniae* under conditions of low and high stringency. Each strain was also screened using a full-length *psp*A probe. Table 64 summarizes the results for each strain under conditions of high stringency. Strain Rx1 is a laboratory derivative of the clinical isolate D39 and consequently, both strains showed identical hybridization patterns and are a single column in Table 64.

Table 64. Summary of hybridization of oligonucleotides with *HINDIII* chromosomal restriction fragments.

	Strains																
Probe	Rx1/D39	WU2	DBL5	DBL6A	A66	AC94	AC17	AC40	AC107	AC100	AC140	DB109	BG9709	BG58C	LB1905	LB2233	LB2006
FL-Rx1[a]	4.0,9.1[b]	3.8	3.7,5.8	3.0,3.4	3.6,4.3	3.6,6.3	3.6,6.3	3.2,3.6	3.2,3.6	4.0,8.0	3.0,4.0	3.3,4.7	3.3,4.7	1.4,3.2 3.6	3.6,5.2	8.2,3.7	4.3,6.4
LSM12	4.0,9.1	3.8	3.7,5.8	3.0,3.4	4.3	[c]	3.6,6.3	3.2,3.6	-	4.0,8.0	4.0	3.3,4.7	2.2,9.6	1.4,3.2,3.6	3.6	1.3,3.7	-
LSM5	4.0	-	-	-	-	3.6,6.3	-	-	-	-	-	-	2.2,9.6	3.6	1.2,2.3,3.6	-	-
LSM3	4.0	3.8	-	-	-	6.3	-	-	-	-	-	-	2.2	3.6	3.6	-	-
LSM4	4.0	-	-	-	-	-	-	-	-	-	-	-	2.2	3.6	3.6	3.7	-
LSM7	4.0,9.1	3.8	3.7	3.0,3.4	3.6	-	-	3.2,3.6	-	-	3.0,4.0	3.3,4.7	2.2,9.6	3.6	3.6,2.3	3.7	-
LSM111	4.0,9.1	3.8	3.7,5.8	3.4	-	6.3	-	3.2	3.6	4.0	4.0	-	2.2	-	5.2	-	-
LSM10	4.0,9.1	3.8	3.7	3.4	3.6,4.3	-	3.6,6.3	3.2	3.6,3.3	4.0	4.0	3.3,4.7	2.2,9.6	3.6,3.2	3.6,5.2	1.3,3.7	4.3,6.4
LSM2	4.0	0	3.7	-	-	3.6	3.6	-	3.6,6.3	4.0	3.0,4.0	4.7	-	-	-	-	4.3

[a] Full-length *pspA* of strain Rx1.
[b] numbers are size in kilobase pairs.
[c] no hybridization observed with corresponding probe.

The only strain which did not have more than one *psp*A-homologous HindIII fragment was WU2, which was previously shown using a full-length *psp*A probe. Even at high stringency, six of the eight probes detected more than one fragment in at least one of the 18 strains (Table 64). LSM7, 10 and 12 hybridized with two fragments in more than one-half of the strains, and the fragments detected by the oligonucleotide probes were identical in size to those detected by the full-length *psp*A probe. Moreover, the same pairs of fragments were frequently detected by probes derived from the 3′ as well as the 5′ region of Rx1 *psp*A. These results suggested that the *Hind*III fragments from different isolates include two separate but homologous sequences, rather than fragments of a single *psp*A gene. Based on the diversity of the hybridization patterns and the size of restriction fragments, it is clear that *psp*A and *psp*C sequences are highly diverse and that these loci have considerable sequence variability as determined by location of *Hind*III recognition sites.

Oligonucleotides which hybridize with a single restriction fragments in each strain were assumed to be specific for *psp*A. At high stringency, LSM3 and LSM4 detected only a single *Hind*III fragment in the strains with which they reacted. Restriction fragments containing homology to LSM3 or LSM4 were the same as those which hybridize with all of the other homologous probes. This suggested that LSM3 and LSM4 specifically detect *psp*A rather than the *pspC* sequence. That LSM3 hybridizes with a single restriction fragment of WU2 further confirmed that this oligonucleotide is specific for *psp*A. Sequences from the portion of the gene encoding the second proline region (LSM111) and the C-terminus (LSM2) appeared to be relatively specific for *psp*A since they generally detect only one of the *Hind*III fragments of each strain.

Oligonucleotides LSM12 and LSM10 were able to detect the most conserved epitopes of *psp*A and generally hybridize with multiple restriction fragments of each strain (Table 65). LSM7 was not as broadly cross-reactive, but detected two *psp*As in 41% of strains including almost 60% of the strains with which it reacts. Thus, sequences representing the leader, first proline region, and the repeat region appear to be relatively conserved not only within *psp*A but between the *pspA* and *pspC* sequences. LSM3, 4, and 5 hybridize with the smallest number of strains of any oligonucleotides (29-35 percent), suggesting that the α-helical domain is the least conserved region within *psp*A. In strains BG58C and L81905 oligonucleotides detect more than two *Hind*III fragments containing sequences with homology to *psp*A. Because of the absence of *Hind*III restriction sites within any of the oligonucleotides it was unlikely that these multiple fragments result from the digestion of chromosomal DNA within the target regions. Also, the additional restriction fragments were detected at high stringency by more than one oligonucleotide. Possibly, in these two strains, there are three or four sequences with DNA homology to some portions of *psp*A. The probes most consistently reactive with these additional sequences are those for the leader, the alpha-helical region, and the proline-rich region.

The oligonucleotides used as hybridization probes were also tested for their utility as primers in the polymerase chain reaction (PCR). Amplification of *pspA* from 14 strains of S. *pneumoniae* comprising 12 different capsular types was attempted with the primers listed in Table 63. LSM2, derived from the 3′ end of *pspA*, were able to amplify an apparent *pspA* sequence from each of 14 pneumococcal strains when used in combination with LSM111, which is within the sequence of *pspA* encoding the proline-rich region. Combinations of LSM2 with primers upstream in *pspA* were variably successful in amplifying sequences (Table 65). The lowest frequency of amplification was observed with LSM112 which was derived from the Rx1 sequence 5′ to the *pspA* start site. This oligonucleotide was not used in the hybridization studies. DNA fragments generated by PCR were blotted and hybridized with a full-length *pspA* probe to confirm homology to *pspA*.

Further evidence for variability at the *pspA* locus comes from the differences in the sizes of the amplified *pspA* gene. When PCR primers LSM12 and LSM2 were used to amplify the entire coding region of PspA, PCR products from different pneumococcal isolates ranged in size from 1.9 to 2.3 kbp. The regions of *pspA* which encode the α-helical, proline-rich, and repeat domains were amplified from corresponding strains and variation in *pspA* appears to come from sequences within the α-helical coding region.

Table 65. Amplification of *psp*A by PCR using the indicated oligonucleotides as 5′ primers in combination with the 3′ - primer LSM2.

5′ - primer	Domain	Amplified/ Tested	Percent Amplified
LSM112	-35 (upstream)	2/14	14
LSM12	leader	8/14	57
LSM3	α-helical	3/14	21
LSM7	proline	12/14	86
LSM111	proline	14/14	100

These studies have provided a finer resolution map of the location of conserved and variable sequences within *pspA*. Additionally, regions of divergence and identity between *psp*A and the *psp*C sequences have been identified. This data confirmed serological studies, and demonstrated that *psp*A and *pspC* sequences are highly variable at the DNA sequence level. The diversity of *Hind*III restriction fragment polymorphisms contained *psp*A and the *pspC* sequence supported earlier data using larger probes that detected extensive variability of the DNA in and around these sequences.

A useful *psp*A-specific DNA probe would identify Rx1 and WU2 *psp*A genes, in which restriction maps are known, and would identify only a single restriction fragment in most strains. Two probes, LSM3 and LSM4, do not hybridize with more than one *Hind*III restriction fragment in any strain of pneumococcus. Both of these oligonucleotides hybridize with Rx1 *psp*A and LSM3 hybridizes with WU2 *psp*A. However, each of these probes hybridize with only four of the other 15 strains. When these probes identify a fragment, however, it is generally also detected by all other Rx1-derived probes. Oligonucleotides from the second proline-rich region (LSM111) and the C-terminus of *psp*A (LSM2) generally identify only one *psp*A-homologous sequence at high stringency. Collectively, LSM111, 2, 3 and 4 react with 16 of the 17 isolates and in each case revealed a consensus DNA fragment recognized by most or all of the oligonucleotide probes.

When an oligonucleotide probe detected only a single DNA fragment it was presumed to be *psp*A. If the probe detected multiple fragments, it was presumed to hybridize with *psp*A. If the probe detected multiple fragments, it was presumed to hybridize with *psp*A and the *pspC* sequence. Based on these assumptions the most variable portion between *psp*A and *pspC* is the region immediately upstream from the -35 promoter region and that portion encoding the α-helical region. The most conserved portion between *psp*A and *pspC* was found to be the repeat region, the leader and the proline-rich region sequences. Although only one probe from within the repeat region was used, the high degree of conservation among the 10 repeats in the Rx1 sequence makes it likely that other probes within the repeat sequences would give similar results.

The portion of Rx1 *psp*A most similar to the *pspC* sequence was that encoding the leader peptide, the upstream portion of the proline-rich region, and the repeat region. The repeat region of PspA has been shown to be involved in the attachment of this protein to the pneumococcal cell surface. The conservation of the repeat region within *pspC* sequences suggests that if these loci encode a protein, it may have a similar functional attachment domain. The conservation of the leader sequence between *psp*A and the *pspC* sequence was also not surprising since similar conservation has been reported for the leader sequence of other proteins from gram positive organisms, such as M protein of group A streptococci (Haanes-Fritz, E. et al., Nucl. Acids Res. 1988; 16: 4667-4677).

In two strain, some oligonucleotide probes identified more than two *psp*A-homologous sequences. In these strains, there was a predominant sequence recognized by almost all of the probes, and two or three additional sequences share homology with DNA encoding the leader, α-helical, and proline region, and they have no homology with sequences encoding the repeat region in the C-terminus of PspA. These sequences might serve as cassettes which can recombine with *psp*A and/or the *pspC* sequences to generate antigenic diversity. Alternatively, the sequences might encode proteins with very different C-terminal regions and might not be surface attached by the mechanism of PspA.

Oligonucleotides which hybridize with a single chromosomal DNA fragment were used as primers in PCR to examine the variability of domains within *psp*A. These results demonstrate that full-length *psp*A varies in size among strains of pneumococci, and that this variability is almost exclusively the result of sequences in the alpha-helix coding region.

EXAMPLE 12 - CLONING OF PspC

Chromosomal DNA from S. *pneumoniae* EF6796, serotype 6A clinical isolate, was isolated by methods including purification through a cesium chloride gradient, as described in Example 8. The *Hind*II-*Eco*RI fragment of EF6796 was cloned in modified pZero vector (Invitrogen, San Diego, CA) in which the Zeocin-resistance cassette was replaced by a kanamycin cassette (shown in Figure 18). Recombinant plasmids were electroporated into *Escherichia coli* TOP10F′ cells [F′

{*lac*I^{q}Tet^R} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *deo*R *recA1 araD*139 Δ*(ara-leu)*7967 *gal*U *gal*K *rps*L *end*A1 *nup*G] (Invitrogen).

The 5′ region of *pspA.Rx1* does not hybridize to *psp*C sequence at high stringencies by Southern analysis. Utilizing both the full-length Rx1 *psp*A probe, and a probe containing the sequence encoding α-helical region of PspA, it was possible to identify which DNA fragment contained *psp*A and which fragment contained the *psp*C locus. The *psp*C locus and the *psp*A gene of EF6796 were mapped using restriction enzymes. After digestion of chromosomal DNA with *Hind*III, the *psp*C locus was localized to a fragment of approximately 6.8 kb. Following a double digest with *Hind*III and *Eco*RI, the *psp*C locus was located in a 3.5 kb fragment. To obtain the intact *psp*C gene of EF6796, chromosomal DNA was digested with *Hind*III, separated by agarose gel electrophoresis, the region between 6 and 7.5 kb purified, and subsequently digested with *Eco*RI. This digested DNA was analyzed by electrophoresis, and DNA fragments of 3.0 to 4.0 kb were purified (GeneClean, Bio101, Inc., Vista, CA). The size-fractionated DNA was then ligated in *Hind*III-*Eco*RI-digested pZero, and electroplated into E. *coli* TOP10F′ cells. Kanamycin-resistant transformants were screened by colony blots and probed with full-length *psp*A. A transformant, LXS200, contained a vector with a 3.5 kb insert which hybridized to *psp*A.

Sequencing of *psp*C in pLXS200 was completed using automated DNA sequencing on an ABI 377 (Applied Biosystems, Inc., PLACE). Sequence analyses were performed using the University Of Wisconsin Genetics Computer Group (GCG) programs supported by the Center for AIDS Research (P30 AI27767), MacVector 5.0, Sequencer 2.1, and DNA Strider programs. Sequence similarities of *psp*C were determined using the NCBI BLAST server. The coiled-coil structure predicted by *psp*C sequence was analyzed using Matcher.

A gene probe for cloning the pspC locus

Two oligonucleotide primers, N192 and C558 (shown in Figure 19), have been used previously to clone fragments homologous to the region of Rx1 *psp*A encoding amino acids 192-588 from various pneumococcal strains. These primers are modifications (altered restriction sites) of LSM4 and LSM2 which were previously shown to amplify DNA encoding the C-terminal 396 amino acids of PspA.Rx1 (Figure 17); this includes approximately 100 amino acids of the α-helical region, the proline rich region, and the C-terminal choline-binding repeat region. Using primers N192 and C558, a 1.2 kb fragment from strain EF6796 was amplified by PCR, and subsequently cloned in pET-9A (designated PRCT135). This insert was then partially sequenced.

Independently, a larger *pspA* fragment from strain EF6796 was made using primers LSM13 and SKH2 (shown in Figure 19) for the purpose of direct sequencing of serologically diverse *pspA* genes.

The LSM13 and SKH2 primer pair result in the amplification of the 5′ end of most *pspA* gene(s) encoding the upstream promoter, the leader peptide, the α-helical, and the proline-rich regions (amino acid -15 to 450) (Figure 20). From the strain EF6796, the LSM13 and SKH2 primers amplified a 1.3 kb fragment (*pspA.EF6796*), which was sequenced. The sequence from pRCT135 and the LSM13/SKH2 PCR-generated fragment *pspA.EF6796* was not identical. The fragment obtained by PCR using primers LSM13 and SKH2 was designated *pspA* based on its location within the same chromosomal location as *pspA.Rx1*. The cloned fragment in pRCT135 was assumed to represent the sequence of the second gene locus, *pspC*, known to be present from Southern analysis. Both genes have significant similarity to the corresponding regions of the prototype *psp*A gene from strain Rx1. The second gene locus was called *pspC*, in recognition of its distinct chromosomal location, not sequence differences from the prototype *pspA* gene.

Analysis of the nucleotide and amino acid sequence of pspC EF6796

To test the hypothesis that pRCT135 represented *psp*C of EF6796, and to further investigate *psp*C, the entire EF6796 *psp*C gene was cloned as a 3.4 kb *Hind*III-*EcoR1* fragment forming pLXS200. DNA sequence of the *psp*C-containing clone pLXS200 revealed an open reading fram of 2782 nucleotides based on the analysis of putative transcriptional and translation start and stop sites (Figure 21). The predicted open reading frame encodes a 105 kDa protein which has an estimated pI of 6.09.

PspA.Rx1 and PspC.EF6796 are similar in that they both contain an α-helical region followed by a proline-rich domain and repeat region (Figure 20). However, there are several features of the amino acid sequence of PspC which are quite distinct from PspA. From comparisons at the nucleotide as well as the predicted amino acid sequence, it is apparent that the region of strong homology between PspC and PspA begins at amino acid 458 of PspC (amino acid 147 of PspA) and extends to the C-terminus of both proteins (positions 899 and 588 respectively). The predicted amino acid sequence of PspC.EF6796 and PspA.Rx1 are 76% similar and 68% identical based on GCG Bestfit program for this region (Figure 22). The nucleotide sequence identity between *psp*C and *psp*A is 87% for the same region. Eight bases upstream of the ATG start site is putative ribosomal binding site, TAGAAGGA. The proposed transcriptional start -35 (TATACA) and -10 (TATAGT) regions are located between 258 to 263 and 280 to 285, respectively (Figure 21). A potential transcriptional terminator occurs at a stem loop between nucleotides 3237 through 3287. The putative signal sequence of PspC is typical of other gram positive bacteria. This region consists of a charged region followed by a hydrophobic core of amino acids. A potential cleavage site of the signal peptide occurs at amino acid 37 following the Val-His-Ala. The first amino acid of the mature protein is a Glu residue.

Other than features similar to all signal sequences, there is no homology in this region between *psp*A and *psp*C. This confirms that *psp*C is present in a separate chromosomal locus from that of *pspA*. The signal sequence and upstream region have striking similarity to the similar regions of *S. agalactiae* ß antigen (accession number X59771). The ßantigen of Group B streptococci is a cell surface receptor that binds IgA. Similarity to the *bac* gene ends with the start of the mature protein of PspC, and the nucleotides are 75% identical in this region. Thus, although *psp*C is in a very similar chromosomal locus to the ß antigen, it is clearly a distinct protein.

The N-terminus of PspC is quite different from the N-terminus of PspA. Prediction of the secondary structure utilizing Chou-Fausman analysis (Chao, P.Y. et al., Adv. Enzymol. Relat Areas Mol. Biol. 1978: 47: 45-148), suggests that the structure of amino acids 16 to 589 of PspC is predominately α-helical. The Matcher program was used to examine periodicity in the α-helical region of PspA. The characteristic seven residue periodicity is maintained by having hydrophobic residues at the first and fourth positions (a and d) and hydrophobic residues at the remaining positions. The coiled-coil region of the α-helix of PspC (between amino acid 32 to 600) has three breaks in the heptad repeat (Figure 23). These disturbances in the 7 residue periodicity occur at amino acids 99 to 104, 224 to 267 and 346 to 350. The α-helical region of PspA has seven breaks in the motif, each break ranging from a few amino acids to 23 amino acids each. In contrast, the three breaks in the coiled-coil motif of PspC involve 5, 43 and 4 amino acids, respectively.

The sequence encoding the α-helical region of PspC contains two direct repeats 483 nucleotides (160 amino acids) long which are 88% percent identical at the nucleotide level. These repeats, which occur between nucleotides 562 to 1045 and nucleotides 1312 to 1795, are conserved both at the nucleotide and amino acid level (amino acids 188 to 348 and 438 to 598) (Figure 24). PspA lacks evidence for any repeats this prominent within the α-helical region. These repeat regions could provide a mechanism for recombination that could alter the N-terminal half of the PspC molecule. Although repeat motifs are common in bacterial surface proteins, a direct repeat this large or separated by a large spacer region is novel. The evolutionary significance of this region is not known. A Blast search of the repeat region and the 267 nucleotide bases between them revealed no sequence with significant homology at the nucleotide or amino acid level. However, one of the structural breaks in the coil-coiled region of PspC is the region between the two repeats. Perhaps some deviation from coiled-coil structure between the two repeats is critical to maintain the α-helical structure.

Previous studies have shown that a major cross-protective region of PspA comprises the C-terminal 1/3 of the α-helical region (between residues 192 and 260 of PspA.Rx1). This region accounts for the binding of 4 of 5 cross-protective immunity in mice. Homology between PspC and PspA begins at amino acid 148 of PspA, thus including the region from 192 - 299. The homology between PspA and the PspC includes the entire PspC sequence C-terminal of amino acid 486. Based on the fact that PspA and PspC are so similar in this region known to be protection-eliciting, PspC is also likely to be a protection-eliciting molecule. Because of close sequence and conformational similarity of the proteins in this region, antibodies specific for the region of PspA between amino acid 148 and 299 should cross-react with PspC and thus afford protection by reacting with PspC and PspA. Likewise, immunization with the PspC would be expected to elicit antibodies cross-protective against PspA. The differences between PspC of strain EF6796 and PspA of strain Rx1 is no greater than the differences between many additional PspAs, which have been shown to be highly cross-protective.

A proline-rich domain exists between amino acid 590 to 652. The sequence, PAPAPEK, is repeated six times in this region. This region is very similar to the proline-rich region of PspA.Rx1 which contains the sequence PAPAP repeated eight times in two proline-rich regions. These two regions of PspA.Rx1 are separated by 27 charge amino acids; no such spacer region is present in PspC.

Many cell surface proteins of other gram positive bacteria contain proline-rich regions. These are often associated with a domain of protein that is predicted to be near the cell wall murein layer when the protein is cell-associated. For example, in M proteins of *S. pyogenes* this domain contains both a Pro- and Gly-rich regions. The fibronectin-binding protein of *S. pyogenes*, *S. dysgalactiae*, and *Staphylococcus aureus* contains a proline-rich region with a three-residue periodicity (pro-charged-uncharged) that is not found in PspA or PspC. An M-like protein of *S. equi* contains a proline-rich region that is comprised of the tetrapeptide PEPK. This region lacks glycine normally found in the proline regions of M-proteins. The last proline repeat region of this molecule is PAPAK, which is more similar to the proline-region of PspA and PspC than it is to M-proteins.

Proline-rich regions of gram positive bacterial proteins have been reported previously to transit the cell wall. The differences in proline-rich regions of proteins from diverse bacteria may reflect differences in protein function or possibly subtle differences in cell wall function. Proline-rich regions are thought to be responsible for aberrant migration of these proteins through SDS-polyacrylamide gels.

The repeat region of PspC is a common motif found among several proteins in gram positive organisms. Autolysin of *S. pneumoniae*, toxins A and B of *Clostridium difficile*, *glucosyltransferases* from *S. downei* and *S. mutans*, and CspA of C. *acetobiltylicum* all contain similar regions. In PspA these repeats are responsible for binding to the phosphatidylcholine of teichoic acid and lipoteichoic acid in cell wall of pneumococci. However, bacterial proteins containing C-terminal repeats are secreted, which may imply either a lost or gained function. Although all of these proteins have similar repeat regions the similarity of the repeat regions of PspA and PspC is much greater than that of PspC to the other proteins (Table 66).

Interestingly, PspC like PspA has a 17 amino acid partially hydrophobic tail. The function of this 17 amino acid region is unknown. In the case of PspA it has been shown that mutants lacking the tail bind the surface of pneumococci as well as PspAs in which the tail is expressed. Presently, it is now known whether PspC is attached to the cell surface or secreted.

PspA and PspC proteins both have α-helical coiled-coil regions, proline-rich central regions, repeat regions, with a choline binding motifs, and the C-terminal 17 amino acid tail. PspA and PspC share three regions of high sequence identity. One of these is a protection-eliciting region present within the α-helical domain. The other two regions are the proline-rich domain and a repeat domain shared with other choline binding proteins and thought to play a role in cell surface association. The similarity throughout most of the structure of the PspA and the PspC molecules raises the possibility that the two molecules may play at least slightly redundant functions. However, the fact that the N-terminal half of the protein is not homologous to any of the α-helical sequence of PspA suggests the PspC and PspA may have evolved for at least somewhat different roles on the cell surface. One of the most striking differences between the two molecules is the single repeat in the α-helical region of PspC. Although neither the exact function of PspA nor of PspC are known, the observation that a major cross-protective region of PspA is highly homologous with a similar region of PspC, raises the possibility that both molecules are protection-eliciting and elicit cross-protective antibodies.

The sequence similarity between the promoter region of the *pspC* gene and the *bac* gene from group B streptococci is very intriguing. It implies that an interspecies recombination event has occurred and, this interspecies recombination has contributed to the evolution of the *pspC*. The *pspC* gene thus has a chimeric structure, being partially like *pspA* and partially like the *6* antigen. In the latter case, all protein similarity is limited to the signal sequence. Similar interspecies recombination events have contributed to the evolution of the genes encoding penicillin binding protein.

Using analogous procedures, a second PspC sequence was isolated from strain D39 of *S. pneumoniae*. Figures 25 to 29 show the sequence data of PspC from strain D39, complete from upstream of the promoter through the proline-rich region. Strain D39 has the same genetic background as strains Rx1, from which *pspA* was sequenced. D39 and Rx1 have the same *pspC* gene based on Southern blot analysis.

The alpha-helical encoding region of the D39 *pspC* gene is one third of the size of the homologous region from the EF6796 *pspC* gene. The proline-rich region of the D39 *pspC* gene was more similar to Rx1 *pspA* than to EF6796 *pspC*. Even so, the two *pspC* genes were 86% identical at the nucleotide sequence, and 67% identical at the amino acid level.

In the alpha-helical sequence of EF6797 *pspC* a strong repeat was observed. This was absent in the *pspC* sequence of D39. The D39 *pspC* sequence also lacks a leader sequence, found in the EF6797 *pspC* sequence.

This data strongly indicates that there is variability in the structure of *pspC*, similar to previous observations for *pspA*. In the case of *pspC*, however, the extent of variability appears to be even greater than that which has been observed for *pspA*.

Table 66.

PERCENT HOMOLOGY OF CHOLINE BINDING REGIONS			
		Percent similarity/identity	
Protein	Organism	PspA	PspC
PspC	*S. pneumoniae*	86/60	100/100
Bacteriophage Cp-1	*S. pneumoniae*	56/30	56/28
LytA	*S. pneumoniae*	57/33	61/32
PspA	*C. perfringens*	64/45	59/42
alpha toxin	*C. novyi*	54/29	57/33
CspB	*C. acetobutylicum*	58/36	61/45

Having thus described in detail certain preferred embodiments of the present invention, it is to be understood that the invention defined by the appended claims is not to be limited by particular details set forth in the above description, as many apparent variations thereof are possible without departing from the spirit or scope thereof.

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WHAT IS CLAIMED IS:

1. An isolated amino acid molecule consisting of residues 1 to 115, 1 to 260, 192 to 588, 192 to 299, or residues 192 to 260 of pneumococcal surface protein A of *Streptococcus pneumoniae*.

2. An isolated DNA molecule consisting of a fragment of pneumococcal surface protein A gene of *Streptococcus pneumonia* encoding the isolated amino acid molecule of claim 1.

3. A PCR primer consisting essentially of the isolated DNA molecule of claim 2.

4. A hybridization probe consisting essentially of the isolated DNA molecule of claim 2.

5. An immunological composition comprising the amino acid molecule of claim 1.

6. An isolated DNA molecule consisting of nucleotides 1 to 26, 1967 to 1990, 161 to 187, 1093 to 1117 or 1312 to 1331, or 1333 to 1355 of a pneumococcal surface protein A gene of *Streptococcus pneumoniae*.

7. A PCR primer consisting essentially of the isolated DNA molecule of claim 6.

8. A hybridization probe consisting essentially of the isolated DNA molecule of claim 6.

9. An isolated DNA molecule consisting of a fragment of a pneumococcal surface protein A gene of *Steptococcus pneumoniae* consisting of a nucleotide sequence (5′ to 3′) selected from

CCGGATCCAGCTCCTGCACCAAAAAC;
GCGCGTCGACGGCTTAAACCCATTCACCATTGG;
CCGGATCCTGAGCCAGAGCAGTTGGCTG;
CCGGATCCGCTCAAAGAGATTGATGAGTCTG;
GCGGATCCCGTAGCCAGTCAGTCTAAAGCTG;
CTGAGTCGACTGGAGTTTCTGGAGCTGGAGC;
CCGGATCCAGCTCCAGCTCCAGAAACTCCAG;
GCGGATCCTTGACCAATATTTACGGAGGAGGC;
GTTTTTGGTGCAGGAGCTGG;
GCTATGGGCTACAGGTTG;
CCACCTGTAGCCATAGC;
CCGCATCCAGCGTGCCTATCTTAGGGGCTGGTT; and
GCAAGCTTATGATATAGAAATTTGTAAC.

10. A PCR primer consisting essentially of at least one isolated DNA molecule of claim 9.

11. A hybridization probe consisting essentially of at least one isolated DNA molecule of claim 9.

12. PCR probe(s) which distinguishes between *pspA* and *pspA*-like nucleotide sequences.

13. PCR probe(s) which hybridizes to both *pspA* and *pspA*-like nucleotide sequences.

14. A PspA extract prepared by a process comprising

growing pneumococci in a first medium containing choline chloride,

eluting live pneumococci with a choline chloride containing salt solution, and

growing the pneumococci in a second medium containing an alkanolamine and substantially no choline.

15. A PspA extract prepared by

growing pneumococci in a first medium containing choline chloride,

eluting live pneumococci with a choline chloride containing salt solution,

growing the pneumococci in a second medium containing an alkanolamine and substantially no choline, and purifying PspA by isolation on a choline-Sepharose affinity column.

16. An immunological composition comprising the extract of claim 14.

17. An immunological composition comprising the extract of claim 15.

18. An immunological composition comprising full length PspA.

19. A method for enhancing immunogenicity of a PspA-containing immunological composition comprising including in said composition the C-terminal portion of PspA.

20. An immunological composition comprising at least two PspAs.

21. The immunological composition of claim 20 wherein the PspAs are from different groups based on RFLP.

22. PCR amplification product from a primer as claimed in claims 3, 7, 10, 12 or 13.

23. An isolated DNA molecule consisting of a nucleotide sequence homologous to a portion of *pspA*.

24. An isolated amino acid molecule comprising pneumococcal surface protein C, PspC, of *Streptococcus pneumoniae* having alpha-helical, proline rich and repeat regions.

25. An isolated DNA molecule comprising a pneumococcal surface protein C gene of *S. pneumoniae* encoding the isolated amino acid molecule of claim 24.

26. A PCR primer consisting essentially of the isolated DNA molecule of claim 25.

27. A hybridization probe consisting essentially of the isolated DNA molecule of claim 25.

28. An immunological composition comprising the amino acid molecule of claim 24.

29. An isolated amino acid molecule of claim 24 having strong homology with pneumococcal surface protein A, PspA, of *S. pneumoniae* from amino acid 458 of PspC, corresponding to amino acid 147 of PspA, extending to a C-terminus of PspC and PspA.

30. An isolated amino acid molecule of claim 24, further comprising a signal sequence consisting essentially of a charged region followed by a hydrophobic core of amino acids.

31. An isolated amino acid molecule of claim 24, wherein the alpha-helical region further comprises a seven residue periodicity and a coiled coil region having three breaks in a heptad repeat.

32. An isolated amino acid molecule comprising pneumococcal surface protein C, PspC, of *S. pneumoniae* having alpha-helical, proline rich and repeat regions, wherein the alpha-helical region comprises a C-terminus having substantial homology with a protection-eliciting region of PspA.

33. An isolated DNA molecule comprising a pneumococcal surface protein C gene of *S. pneumoniae* encoding the isolated amino acid molecule of claim 32.

34. A PCR primer consisting essentially of the isolated DNA molecule of claim 33.

35. A hybridization probe consisting essentially of the isolated DNA molecule of claim 33.

36. An immunological composition comprising the amino acid molecule of claim 32.

37. An isolated amino acid molecule of claim 24, further comprising a 17 amino acid, partially hydrophobic tail.

38. An isolated amino acid molecule of claim 32, further comprising a 17 amino acid, partially hydrophobic tail.

39. An isolated amino acid molecule of claim 24, further comprising an epitope of interest.

40. An isolated amino acid molecule of claim 32, further comprising an epitope of interest.

41. An immunological composition comprising the amino acid molecule of claim 39.

42. An immunological composition comprising the amino acid molecule of claim 40.

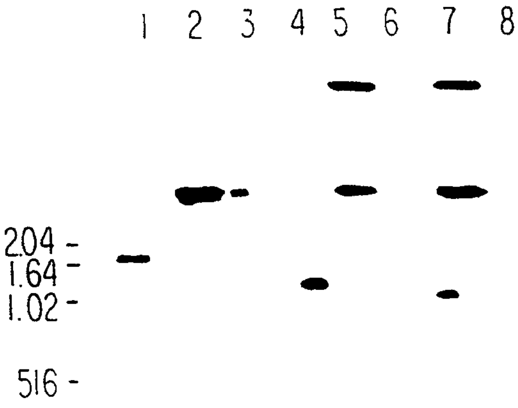


FIG. 1A

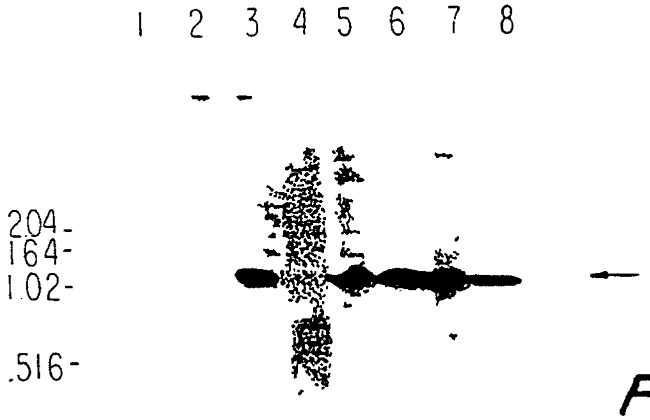


FIG. 1B

FIG. 2

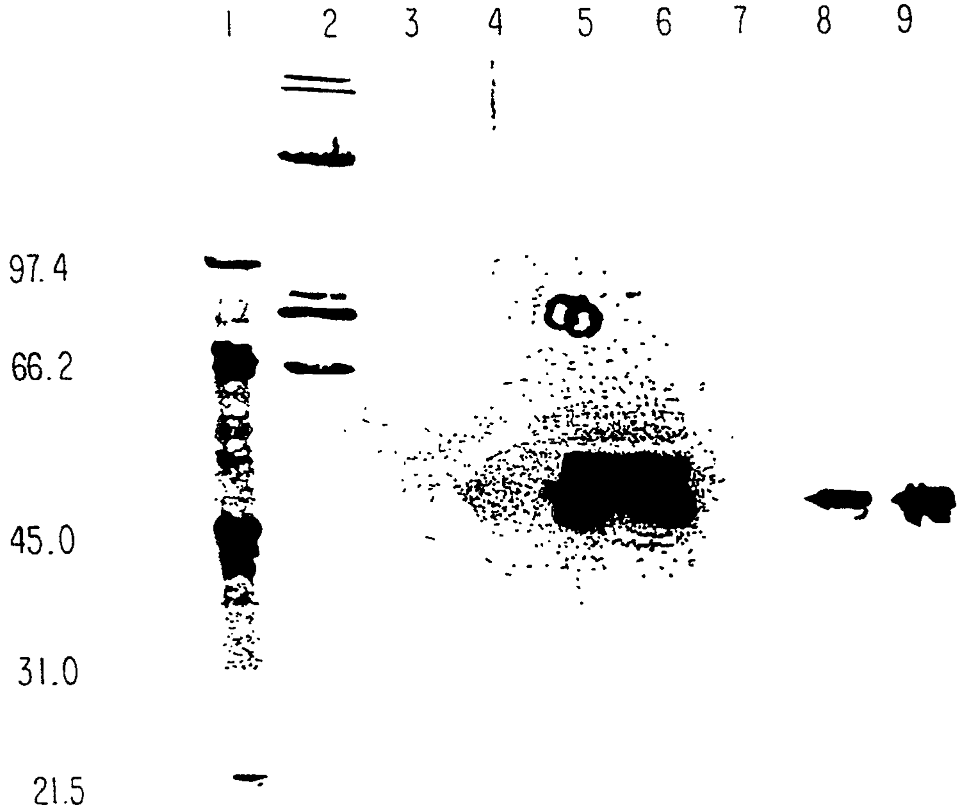
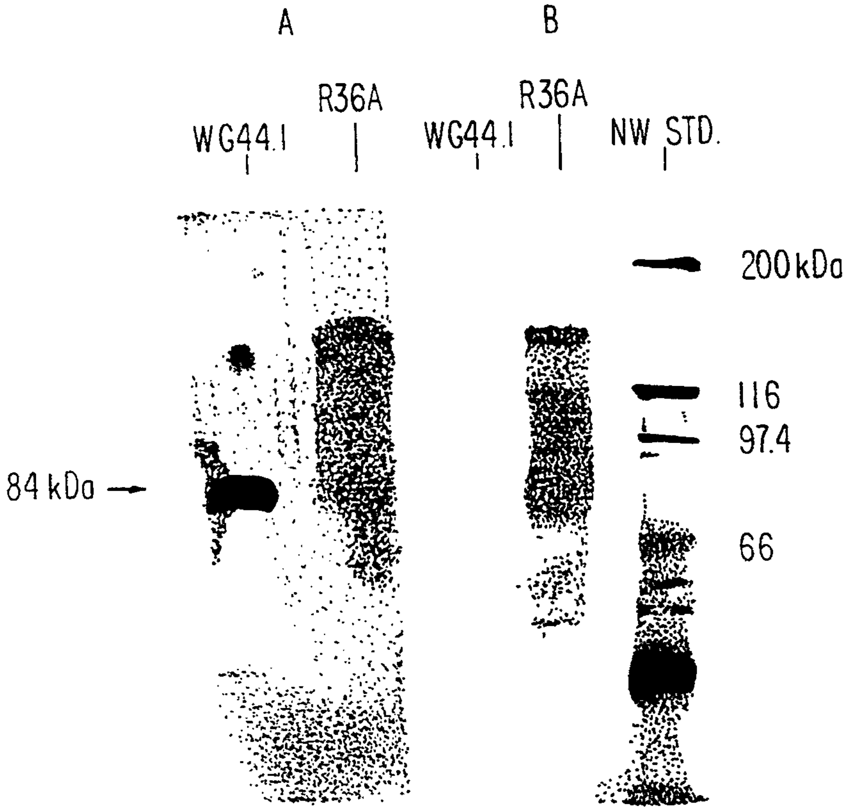
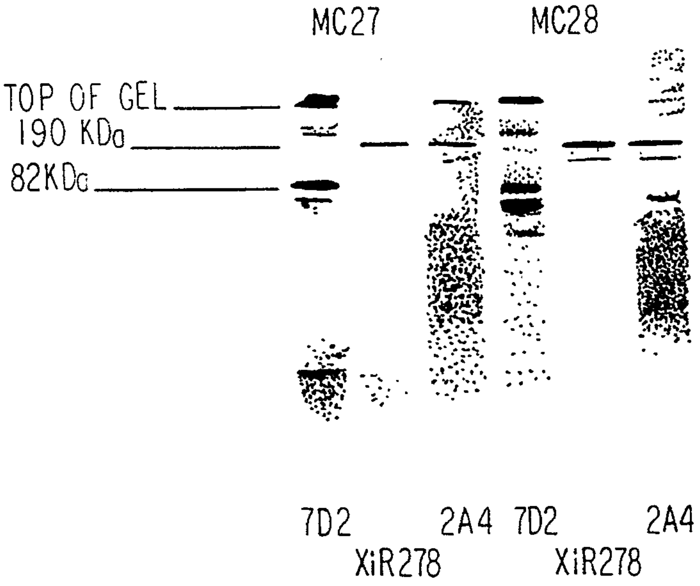


FIG.3

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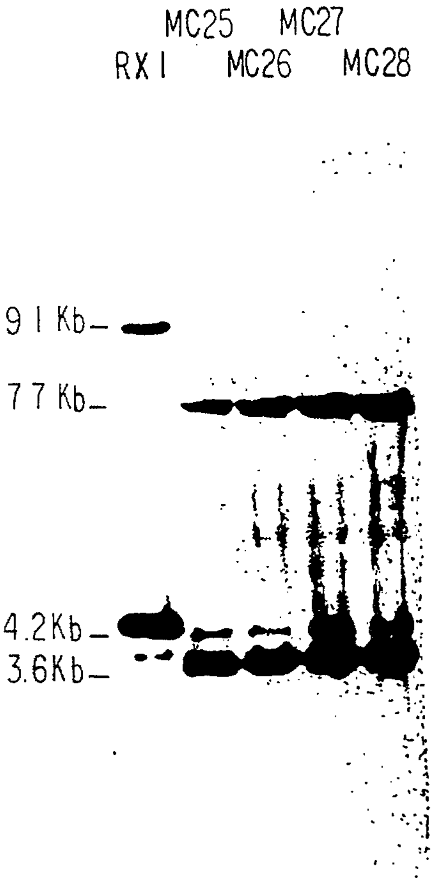
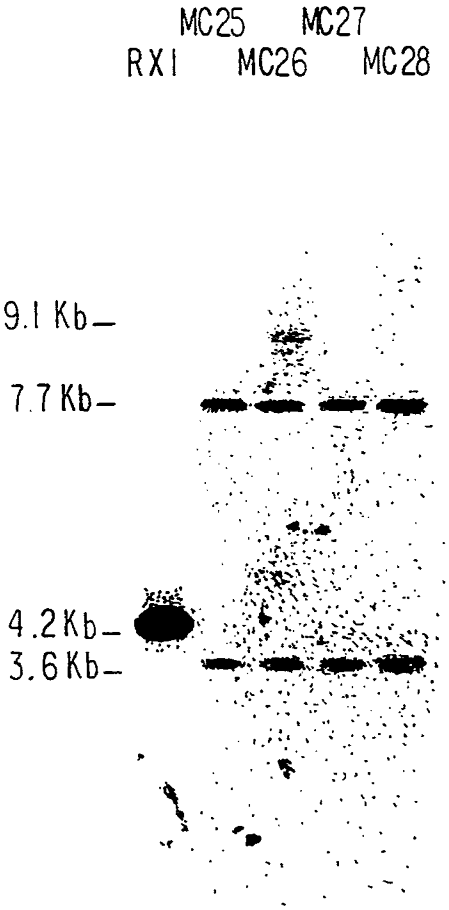
MC28
TOP OF GEL
190 KDa
82KDa
7D2
2A4
7D2
2A4
XiR278
XiR278

FIG. 4



pLSMpspA13 / 2

FIG. 5A



pLSMpspA12 /6

FIG. 5B

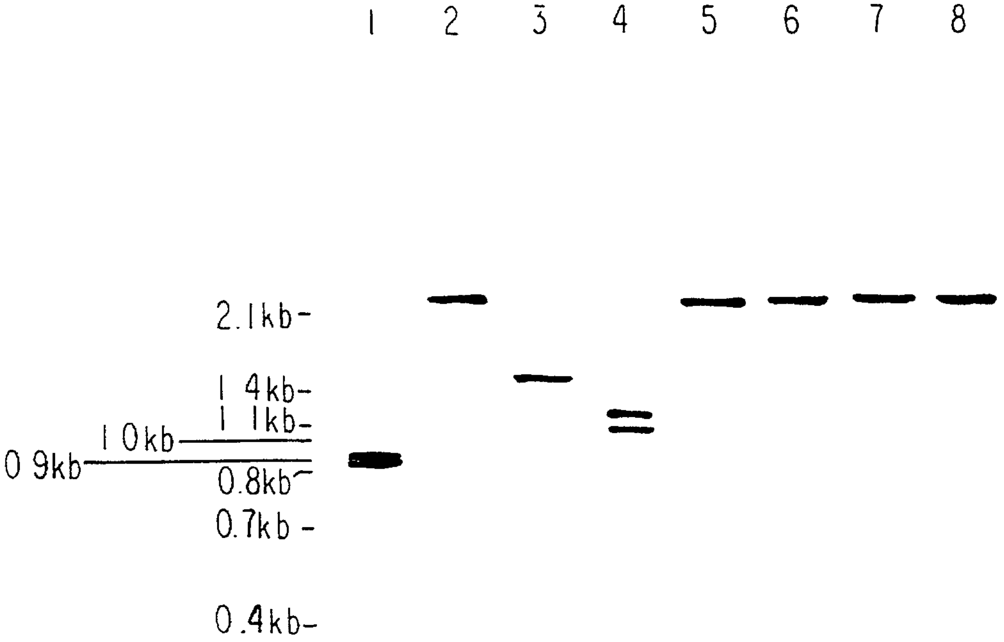
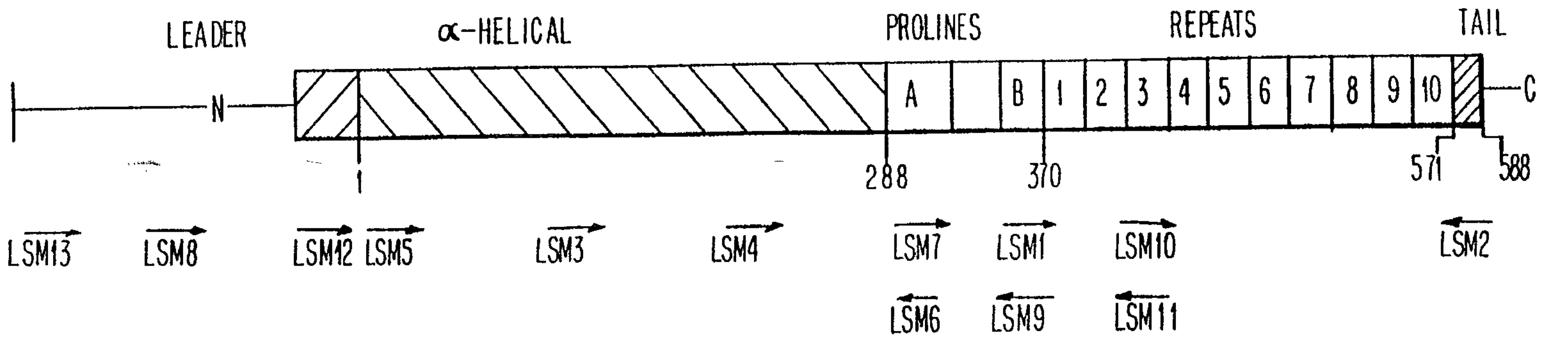


FIG. 6


LEADER
α-HELICAL
PROLINES
REPEATS
TAIL
N
C
A
B
1
2
3
4
5
6
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8
9
10
1
288
370
571
588
LSM13
LSM8
LSM12
LSM5
LSM3
LSM4
LSM7
LSM6
LSM1
LSM9
LSM10
LSM11
LSM2

FIG.7

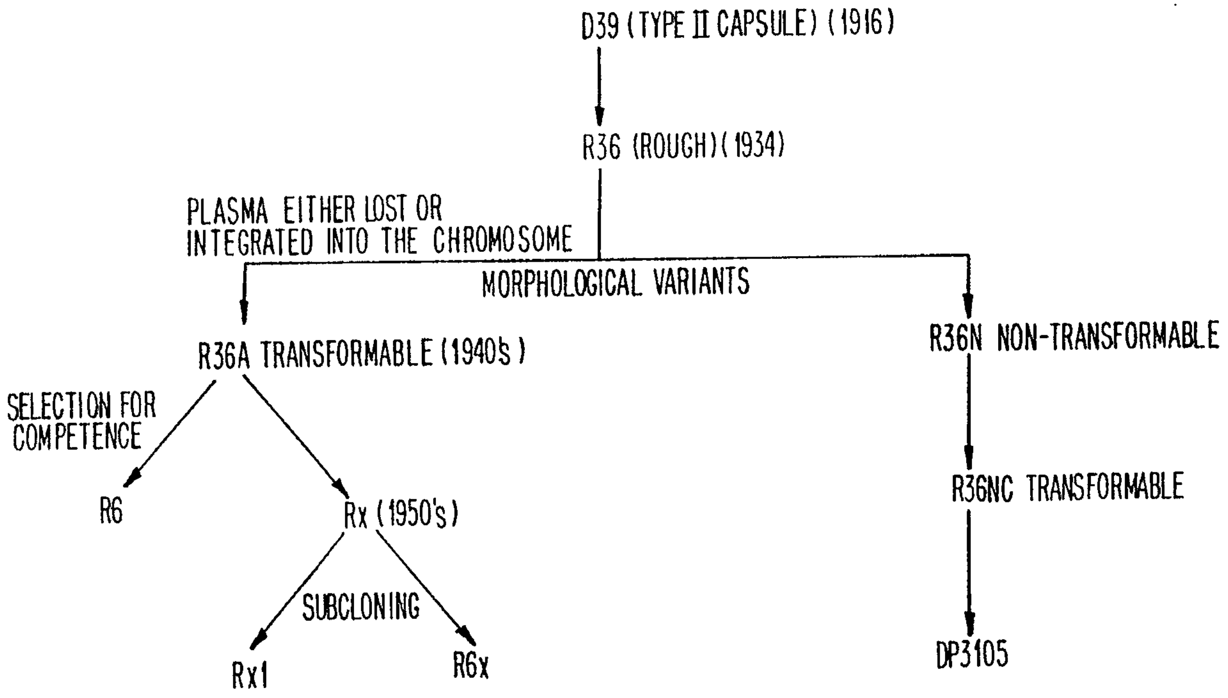
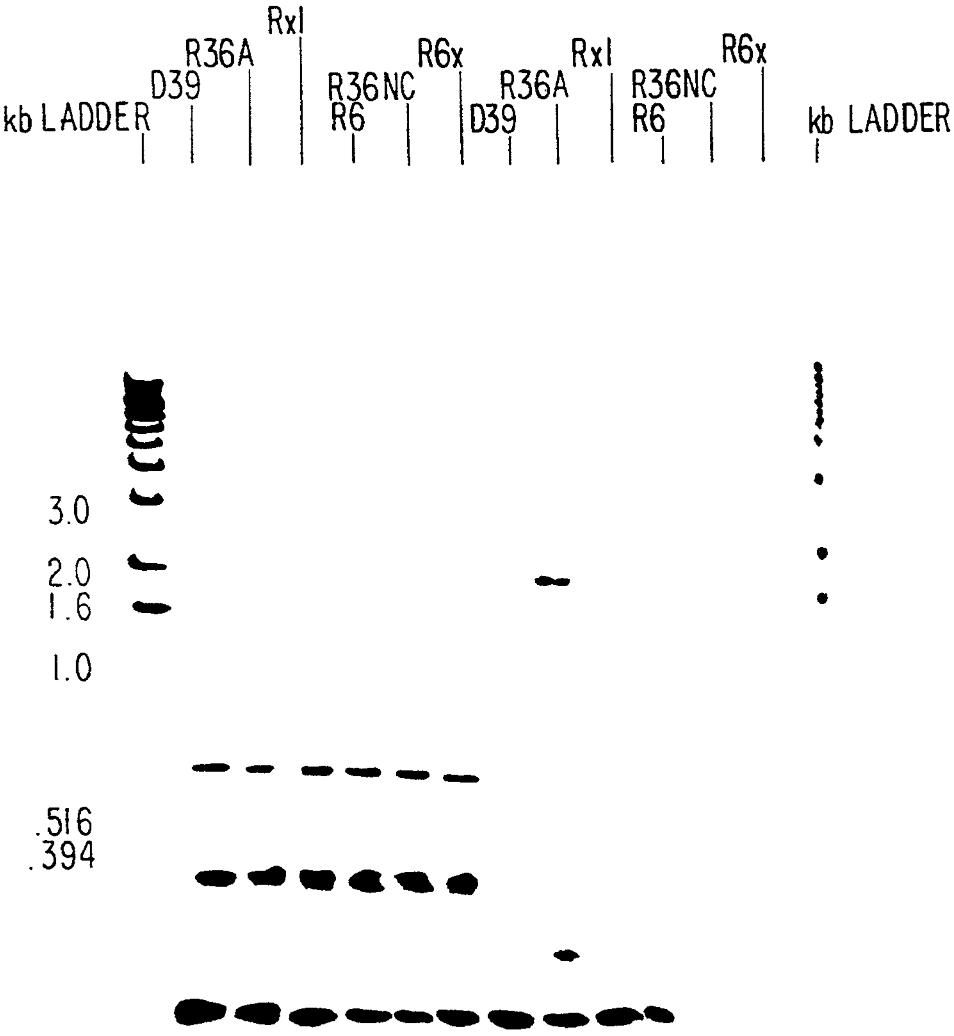

D39 (TYPE II CAPSULE) (1916)
R36 (ROUGH) (1934)
PLASMA EITHER LOST OR INTEGRATED INTO THE CHROMOSOME
MORPHOLOGICAL VARIANTS
R36A TRANSFORMABLE (1940's)
R36N NON-TRANSFORMABLE
SELECTION FOR COMPETENCE
R6
Rx (1950's)
R36NC TRANSFORMABLE
SUBCLONING
Rx1
R6x
DP3105

FIG.8

FIG. 9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R36A
RxI
R6
R36NC
R6x
D39
R36A
RxI
R6
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R6x
kb LADDER
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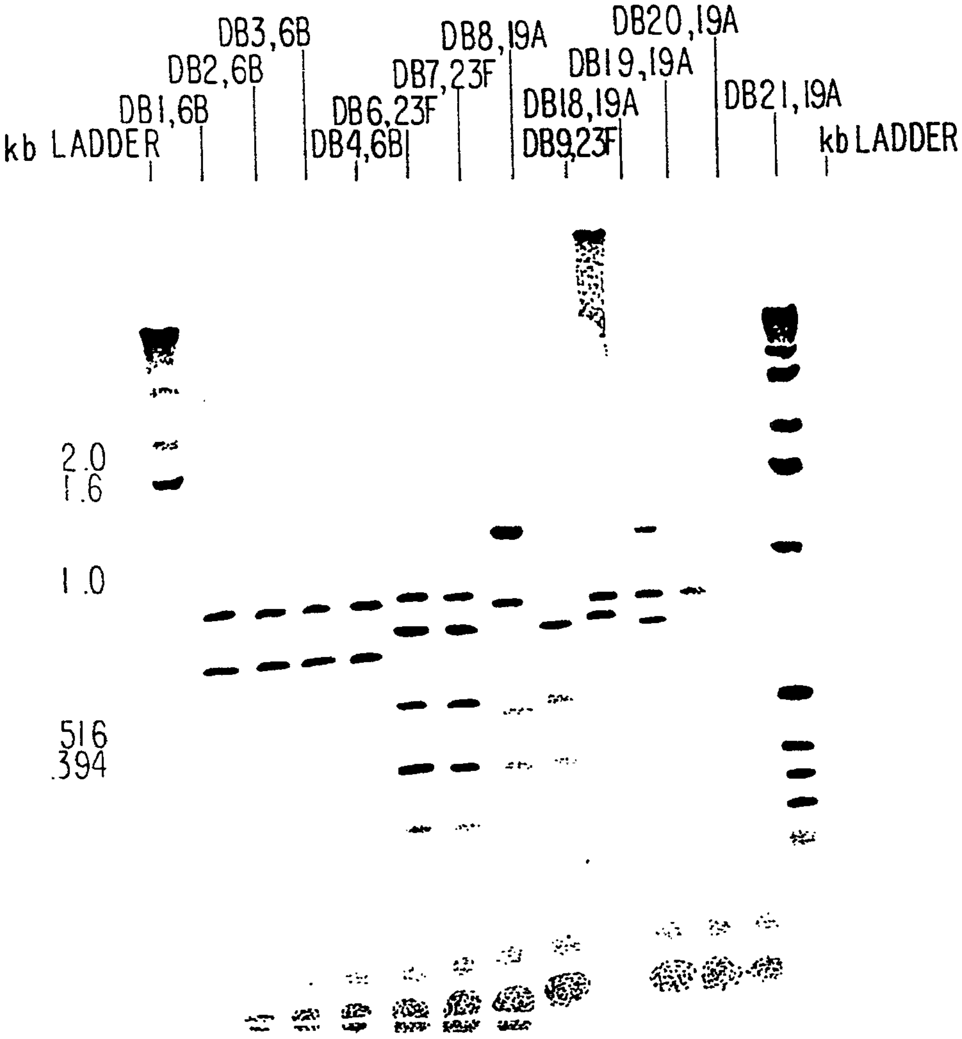
2.0
1.6
1.0
.516
.394

FIG. 10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DB2,6B
DB3,6B
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DB9,23F
DB18,19A
DB19,19A
DB20,19A
DB21,19A
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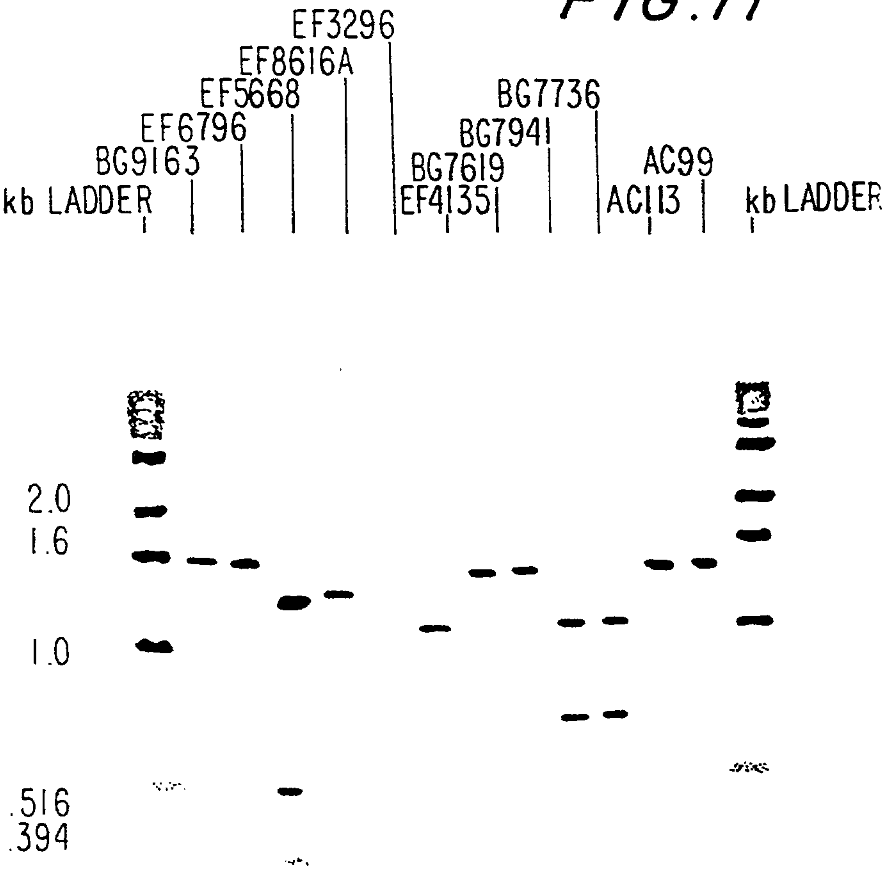
1.6
1.0
516
394

FIG. 11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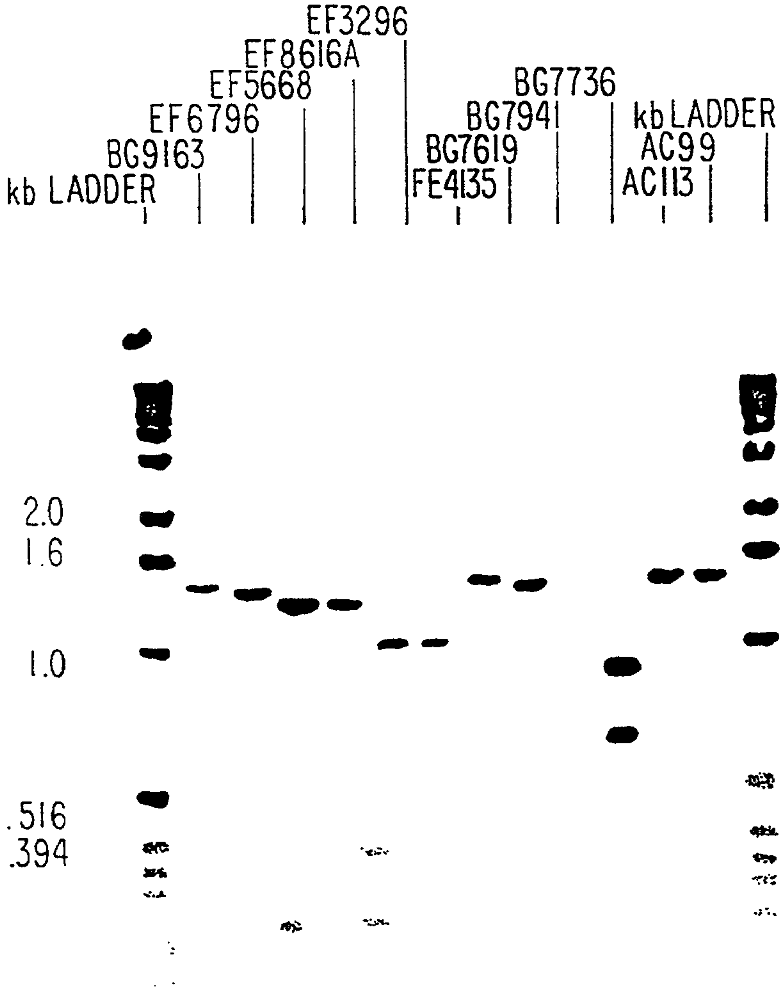
1.6
1.0
.516
.394

FIG. 12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FE4135
BG7619
BG7941
BG7736
AC113
AC99
kb LADDER
2.0
1.6
1.0
.516
.394

AMINO ACID SEQUENCES IN THE NH2-TERMINAL END OF DIFFERENT PSPA GENES. Gap inserted to maximize alignment with related PspA sequences).

```
AtCC6303     MNKKKMILTS LASVAILGTG FVASPPTLVR AEESPQVVEK SSLEKKYEEA
             KAKADTAKKD YETAKK...K AEDAQKKYDE DQKKTEDKAK A.VKKVDEER
             QKAILAVQKA YVEY....RE AKDKASAEKQ IAEAKRKT.. ..........
             .......... .......... .......... .......... ..........
             .......... ..........
Ac94...      MNKKKMILTS LASVAILGAG LVTAQPTLVR AEEAP.VASQ SKAEKDYDTA
             KRDAENAKKA LEEAKR.... ...AQKKYED DQKKTEEKAK E.EKQASEAE
             QKANLQYQLK LREYIQ..KT GDRSKIQTEM EEAEKKHKTA KAEFDKVRGT
             VIPSAARV.. .......... .......... .......... ..........
             .......... ..........
Bg11703pro   MNKKKMILTS LASVAILGAG LVTSQPTLVR AEEAP.VASQ SKAEKDYDAA
             VKKSEAAKKA YEEAKK...K AEDAQKKYDE DQKKTEEKA. ENEKKAAADL
             TEATEVHQKA YVRYSGSNEQ KIKNFKILAI .......... ..........
             .......... .......... .......... .......... ..........
             .......... ..........
Bg7322pro    MXKKKMILTS LASVAILGAG XVASQPTXVR AEDAP.VANQ SQAEKDYXAA
             XXKSEAAKKX YXXAKKVLAE AEAAQKXXED XQKKPEEKA. EKAKAASEEI
             VKATEEVQXA A......... .......... .......... ..........
             .......... .......... .......... .......... ..........
             .......... ..........
```

FIG. 13A

```
Bg7561pro    MNKKKMILTS LASVAILGAG LVTSQPTLVR AEEAP.GASQ SKAEKDYXAA
             XKKSEAAKKA YEEAKK...K AEDAQKKYDE GQKKTEEKA. RKAEEASKEL
             AKATSEVQNA YVKYQGVQRN SRLNEKERKK QLAEIDEEIN KAKQIWNEKN
             EDFKKVREEV IPEPTELAKD QRKAEEAKAE EKVAKRKYDY ATLKVALAKS
             YVEAEEAXL. ..........
Bg8090pro    MNKKKMILTS LASVAILGAG LVTSQPTFVR AEEAP.VASQ PKAEKDYDPA
             GKKSEAATKA YEDAKP...T AEDAQKKYDE AQKKPDAER. ..........
             .......... .......... .......... .......... ..........
             .......... .......... .......... .......... ..........
             .......... ..........
Bg8743pro    MNKKKMILTS LASVAILGAG LVASQPTVVR AEEAP.VAKQ SQAERDYDAA
             MKKSEAAKKE YEEAKKDLEE AKAAQKKYGG DPKKTGEETK LVPK.ADGER
             PKANVAVPKA YLKLREAQEQ LNQSPNNKKN SAQQKLKDAL AHIDEVTLNQ
             KEAEA..... .......... .......... .......... ..........
             .......... ..........
Bg8838pro    MNKKKMILTS LASVAILGAG LVTSQPTVVR AEESP.VASQ SKAEKDYDAA
             VKNATAAKKA AEDAHRALDE AKAAQKNYDE DQKKPEEKAK EVPKAPAEE.
             .......... .......... .......... .......... ..........
             .......... .......... .......... .......... ..........
             .......... ..........
```

FIG. 13B

```
Bg9163pro    MNKKKMILTS LASVAILGAG LVASQPTLVR AEDAP.VANQ SQAEKDYDAA
             MKKSEAAKKE YEDAKKVLAE AEAAQKKYED DQKKTEEKA. ENANAASEEI
             AKATEEVH.. .......... .......... .......... ..........
             .......... .......... .......... .......... ..........
             .......... ..........
Bg9739pro    MNKKKMILTS LASVAILGAG LVASSPTVVR AEEAP.VASQ SKAEKDYDTA
             KRDAENAKKA LEEAKR.... ...AQEKYAD YQRRIEEKAA K.ETQASLEQ
             QEANKDYQLK LKKYLDGRNL SNSSVLKKEM EEAEKKDKEN QAEFNKIRRE
             IVVPNPQELE MARRKSEVVK ATESGLVTRV EEAEKNVTDA RQKLVLKCNE
             VVLQAXXAEL ESGGHKLEPK
Db11pro      MNKKKMILTS LASXAILGAG LVASQPTVVR AEEAP.VASQ SKAEKDYDAA
             KRDAENAKKA LEEAKR.... ...AQKXXED DQKKTEEKAK X.DXQASEAE
             QKANLXYQLL LQKYVSESDG KKKKEXEXXA DAAKKEIELK XADLXKIXQE
             .......... .......... .......... .......... ..........
             .......... ..........
Db15pro      MNKKKMILTS LASVAILGAG LVASQPTVVR AEEAP.VASQ SKAEKDYDAA
             VEKSKAAEED LE.......E AEAAQRKYDE DQKKSEENEK E.TEEASERQ
             QAATLKYHLE SXEFLNYFQD NHR....... .......... ..........
             .......... .......... .......... .......... ..........
             .......... ..........
Db16aapro    MNKKKMILTS LASVAILGAG LVASPPTVVR AEEAP.VASQ SKAEKDYDTA
             KRDAENAKKA LEEAKR.... ...AQEKYAD YQRRIEEKAA K.ETHASLEQ
             QEANKDYQLK LKKYLDGRNL SNSSVLKKEM EEAEKKDKEK PAEFNKIRRE
             IVVPNPQELE MARRKSEVAK TKESGLVKRV EEAEKKVTEA RPKLDAERAK
             EVVLQAQIA. ..........
```

FIG. 13C

```
Db16apro     MNKKKMILTS LASVAILGAG LVASPPTVVR AEEAP.VASQ SKAEKDYDTA
             KRDAENAKKA LEEAKR.... ...AQEKYAD YQRRIEEKAA K.ETHASLEQ
             QEANKDYQLK LKKYLDGRNL SNSSVLKKEM EEAEKKDKEK QAGL......
             .......... .......... .......... .......... ..........
             .......... ..........
Ef10197pro   MNKKKMILTS LASVAILGAG LVTSQPTLVR AEESP.VASQ SKAEKDYDAA
             KRDAENAKKA LEEAKR.... ...AQEKYAD YQRRIEEKAA K.EQQASLEQ
             QEANKDYQLK LKKYLDGRNL SNSSVLKKEM EEAEKKDKEK QAEFNKIRRE
             IVVPNPQELE MARRKSEVVK AKESGLVKRV EEAEKKVTEA RQKLDAERAK
             EVVLQPTR*V ENEVHKLXQK
Ef3296pro    MNKKKMILTS LASVAILGAG LVTSQPTFVR AEESPQVVEK SSLEKKYEEA
             KAKADTAKKD YETAKK...K AEDAQKKYED DQKRTEEKAR K.EAEASQKL
             IDVALVVQNA YKEY....RE VQNQRSKYKS DADYQKKLTE VDSKIEKARK
             EQQDLQNNFN EVRAVVAPDP TCVGXDXR.. .......... ..........
             .......... ..........
Ef6796pro    MNKKKMILTS LASVAILGAG XVTSQPTXVR AEEAPQVVEK SSLEKKYEEA
             KAKYDAAKKD YDEAKK...K AAEAQKKYEE DQKKTEEKAE K.AKAASEEI
             AKATEEVQKA VLDYITAIRN HNDSGKTSAE EAENKAKERD YCCAGKKFDP
             IQTPFVASLT QMIL...... .......... .......... ..........
             .......... ..........
L81905pro    MNKKKMILTS LASVAILGAG LVASSPTVVR AEEAP.VASQ SKAEKDYDTA
             KRDAENAKKA LEEAKR.... ...AQEKYAD YQRRIEEKAA K.ETQASLEQ
             QEANKDYQLK LKKYLDGRNL SNSSVLKKEM EEAEKKDKEN QAEFNKIRRE
             IVVPNPQELE MA........ .......... .......... ..........
             .......... ..........
```

FIG. 13D

```
Rx1pro     MNKKKMILTS LASVAILGAG FVASQPTVVR AEESP.VASQ SKAEKDYDAA
           KKDAKNAKKA VEDAQKALDD AKAAQKKYDE DQKKTEEKA. ALEKAASEEM
           DKAVAAVQQA YLAYQQATDK AAKDAADKMI DEAKKREEEA KTKFNTVRAM
           VVPEPEQLAE TKKKSEEAKQ KAPELTKKLE EAKAKLEEAE KKATEAKQKV
           DA........ ..........
Wu2pro     MNKKKMILTS LASVAILGAG LVASQPTLVR AEESP.VASQ SKAEKDYDAA
           VKKSEAAKKA YEEAKKALEE AKVAQKKYED DQKKTEEKA. ELEKEASEAI
           AKATEEVQQA YLAYQRASNK A..EAAKMIE EAQRRENEAR AKFTTIRTTM
           VVPEPEQLAE TKKKAEEAKA KEPKLAKKAA EAKAKLEEAE KKATEANPQV
           DA........ ..........
Ef5668pro  MNKKKMILTS LASVAILGAG FVASSPTFVR AEEAP.VANQ SKAEKDYDAA
           VKKSEAAKKD YETAKK...K AEDAQKKYDE DQKKTEAKAE K.ERKASEKI
           AEATKEVQQA YLAYLQASNE SQRKEADKKI KEATHAKMRR TCNLTIEFEQ
           QLYFLNQVSY LRLRKKQKRQ QKKQKYLRKN LKRQLKRYKY RKIKYLNKML
           KTKRKL.... ..........
Bg6692pro  MNKKKLIVTS LASVAILGAD SVTSPPALVR ADEASLIASQ SKAEKDYDAA
           KKDAKNAKKA VEDAQKALDD AKAAQKKYDE DQKKTEKKAA AV.KKIDEEH
           QAANLKSQQA LVEFLAAQRE GNPKKKKAAQ ATLEEAENAE KETK......
           .......... .......... .......... .......... ..........
           .......... ..........
```

FIG.13E

```
Ac122pro    MNKKKMIKTS LASAAIFGAX SETSQPTRVR PVEAPE.ARH PKVDKYYDAE
            ADEY...... .......... .......... .......... ..........
            .......... .......... .......... .......... ..........
            .......... .......... .......... .......... ..........
            .......... ..........
A66pro      MNKKKMILTS LASVAILGAG FGCVSAYSCK SRRISRS*SA *SSQRL....
            .......... .......... .......... .......... ..........
            .......... .......... .......... .......... ..........
            .......... .......... .......... .......... ..........
            .......... ..........
L82013pro   MNKKKMILKS LASAAISGAX LVXPQPTLVR AEESP.AASQ SHPEQDYDXX
            XXLCXXLXHQ PSXGRTLLXX XXSXPXSPTP XXXXXXPXSX LTXLXPLXXX
            LKPFPLPXSX PXPPXPPXSP PSPPPRPXLY XXPPXPXPXL SLXLIPFLLL
            XLPPPXXXLP HLXSPPXPXL PPSPTPX... .......... ..........
            .......... ..........
```

FIG. 13F

SEQUENCES IN THE CENTRAL REGION - (Includes Carboxy-terminus of alpha-hel and region and some of the proline-rich region. Gaps are inserted to maximize alignment related PspA sequences.)

```
 30 336
0922134c     .......... .......... .......... .......... ..........
             .........L KEIDESDSED YLKEGLRAPL QSKLDTKKAK LSKLEELSDK
             IDELDAEIAK LEVQLKDAEG NNNVE..... A.YFKEGLEK TTAEKKAELE
             KAEADLKKAV DEPETPAPA. ......PQPA PAPEKPAE.. .........K
             PAPAPAP... EKPAPAPE.. ....K.PAEK PAEKPAEEPA EKPAPAPEK.
             .......... ....PAPTPE .KPAPTPETP KTGWKQENGM ..........
             .......... .......... .......... ......
Atcc6303c    .......... .......... .........V LDXTIAEGKA GIAAXPPNID
             KT....PKDL EDSGLGLEKV LATLDPGGET PDGLDKEASE DSNIGALPNQ
             VSDLENQVSE LDREVTRLPS DLKDTEGNNV GDYVKGGLEK ALTDEKVGLN
             NTPKALDTAP KALDTALNEL G.PDGDEEET PAPAPKPE.. .......QPA
             EQP.....K. .......... ....PAPAPK PEKTDDQQAE EDYARRSEEE
             YNRLPQQQPP KAEK..PAPA PKPEQPVPAP .......... ..........
             .......... .......... .......... ......
Ac122c       .......... .......... .......... .......GGW SWR*ILLARP
             DRLAARQAEL AQKQTELGKL LDSLDPEGKT QDELDKEAGE ....AELDKK
             ADGLPNKVSD LEKEISNLEI LLGGADSEDD T....AALPN KLATKKAELE
             KTQKELDAAL NELG...... ..PDGDEEET PAPAPQPE.. .........Q
             PAPAPKPEQ. .......... ....PTPAPK PEQPTPAPKP EQ..PAP...
             ........AP KPEQ..PAPA PKPEQPAPAP KP.EQPTPGP KIE.......
             .......... .......... .......... ......
```

FIG. 13G

```
A66c     .......... .......... .......... .......... ..........
         .......... .......... .......... .......... ..........
         .......... .......... .......... .........E LLLLEKAGLG
         KAGADLKEAV NEPGESAGEP SQPEEPAEEA PAPEQPTEPT ..........
         .....QPEEP AGETPAPKPE K...PAGQPK AEKTDDQQAE EDYARRSEEE
         YNRLTQQQPP KAEKPAPA.. PQPEQPAPAP K......... ..........
         .......... .......... .......... ......
Ac94c    .......... .......... .......... .......... ..........
         .........L KEIDESDSED YVKEGLRVPL QSELDVKQAK LLKLEELSDK
         IDELDAEIAK NLKKDVEDFQ NSGGGYS... .ALYLEAAEK DLVAKKAELE
         KTEADLKKAV NEPEKPAEE. .......... ..PENPAP.. .......APK
         PAPAPQP... ...EKPA... ....PAPAPK PEKSADQQAE EDYARRSEEE
         YNRLTQQQPP KAEKPAPAPV PKPEQPAPAP KSR....... ..........
         .......... .......... .......... ......
Bg8090c  VXLDRGPAEA AVKEQVDSPP QQLAD*VKEI STRGKFLGGA ATEDETSALP
         NKITAKQAEL AKKQTELEKL LDNLDPEGKT QDELDKEAAE ....AELDKK
         ADELPNKVAD LEKEISNLEI LLGGADPEDD T....AALPN KLATKKAEFE
         KTPKELDAAL NELG...... ..PDGDEEET PA........ ..........
         PAPAPKPEQ. .......... ....PAPA.. .....PAPKP EQPAPAP...
         ........AP KPEQPAPAPA PKPEQPTPAP K......... ..........
         .......... .......... .......... ......
```

FIG. 13H

```
Bg8743c    .......... .......... .......... .......... ..........
           .........L KEIDESDSED YIKEGLRAPL QSKLDAKKAK LSKLDELSDK
           IDELDAEIAK LEKDVGDFPN SDGEQ..... AGQYLVAAEK DLDAKEAELG
           NTGADLKKAV DEPETPAPA. ......PAPK PAPAPAPT.. .........P
           EAPAPA.... PKPAPAPK.. ....PAPAPK PAPAPKPAPA PKPAPAPK..
           .......... ..PAPAPKPE RT........ .......... ..........
           .......... .......... .......... ......

Bg9163c    .......... .......... .......... .......... .......END
           GVQRTRKRAP KRIMSLSQKV XLKXVCRAPL QSKLDAQKAE LLKLEELSGK
           IEELDAEIAE LEVQLKDAEG NNNVE..... A.YFKEGLEK TTAEKKAELE
           XAXADLKKAV DEPETPAPA. ......PAPA PAP....... .........A
           PAPAPA.... PAPAPAPK.. ....PAPAPK PAPAPAPAPA PKPAPAPK..
           .......... ..PAPAPAPA PKPEKPAEKP APAPKPETXK TYG.......
           .......... .......... .......... ......

Bg9739c    .......... .......... .......... .......... ..........
           .........L KEIDESDSED YVREGFRAPL QSELDAKQAK LSKLEELSDK
           IDELDAEIAK LEKDVEDFQN SDGEQ..... AGQYLAAAGE DLIAKKAELE
           KAEADLKKAV DEPETPAPA. ......PA.. PAPAPAPT.. .........P
           EAPAPAPAPA PKPAPAPK.. ....PAPAPK PAPAPKPAPA PKPAPAPK..
           .......... ..PAPAPAPA PKPEKPAEKP APAPKPE... ..........
           .......... .......... .......... ......
```

FIG. 13I

```
Ef1019c     .......... .......... .......... .......... ..........
            .........L KEIDESDSED YVKEGFRAPL QSELDAKQAK LSKLEELSDK
            IDELDAEIAK LEDQLKAAEE NNNVE..... .DYFKEGLEK TIAAKKAELE
            KTEADLKKAV NEPEKPAEEP SQPEKPAEEA PAPEQPTEPT QPEKPAEQPQ
            PAPAPQPEKP AEETPAPKPE K...PAEQPK AEKPADQQAE EDYARRSEEE
            YNRLTQQQPP KAEKPAPA.. PKTK...... .......... ..........
            .......... .......... .......... ......
Ef3296c     .......GGS ALDQEAAAPP HQVADLEKQI TGPEIFLGGA DPEADIAARP
            NELAAKQAEL AQKPTGLEKL LDSLDPGGKT QDELDKEAGE ....AELDKK
            ADELPNKVAD LEKEISNLEI LLGGADSEDD T....AALPN KLAXKXAELE
            KTQKELDAAP NELG...... ..PDGDEEET PAPAPQPE.. .........Q
            PAPAPKPEQ. .......... ....PAPAPK PEQPAPAPKP EQ..PAP...
            ........AP KPEQ..PAPA PKPEQPAKPE KPAEEPTQPE KPATPKT...
            .......... .......... .......... ......
Ef6796xc    .......... .......... .......... .......... ..........
            .......... .......... .......... .......... .........R
            VRAL..KVAE FGVQLRDAGG SNNVG..... A.YFKEGLEE TTAEXEAGLG
            KAEADLKKAV DEPET..... .......... PAP....... .........A
            PAPAPA.... PAPAPAPK.. ....PAPAPK PAPAPAPAPA PKPAPAPK..
            .......... ..PAPAPAPA PKPEKPAEKP APAPKPETPK T.........
            .......... .......... .......... ......
```

FIG. 13J

```
Db15c     .......... .......... .......... .......... ..........
          .........L KDIDESDSED YAKEGLRAPL QSELDTKKAK LLKLEELSGK
          IEELDAEIXE LEVQLKDAEG NNNVE..... A.YFKEGLEK TTAEKKAELE
          KAEADLKKAV DEPETPAPA. ......PAPA PAPAPTPE.. .........A
          PAPAPA.... PKPAPAPK.. ....PAPAPK PAPAPKPAPA PKPAPAPKPA
          PAPAPAPAPK PAPAPAPAPA PKPEKPAEKP APAPKPETPK TGWKQENGM.
          .......... .......... .......... ......

L81905c   .......... .......... .......... .......... ..........
          .........L KEIDESDSED YVKEGFRAPL QSELDAKQAK LSKLEEXSDK
          XDELDAEIAK LEKDVEDFKN SDGEQ..... AGQYLAAAEE DLIAKKAXLE
          KAEADLKKAV DEPETPAPA. ......PA.. PAPAPAPT.. .........P
          EAPAPA.... ..PAPAPK.. ....PAPAPK PAPAPKPAPA PKPAPAPK..
          .......... ..PAPAPAPA PKPEKPAA.. .......... ..........
          .......... .......... .......... ......

Rct115c   .......... .......... .......... .......... ..........
          .......... .......... .......... .......... ..........
          .......... LKEIDESDVE VKKAELELVK EEAKEPRNEE KVKQAKAEVE
          SKKAEATRLE KIKTDRKKAE EAKRKAAEED KVKEK..... ..........
          ..PAPKPEN. .......... ....PAEQPK AEKPADQQAE EDYARRSEEE
          YXRLTQQQPP KTEKPAQPST PKT....... .......... ..........
          .......... .......... .......... ......
```

FIG. 13K

```
Rct121c     .......... .......... .......... .......... ..........
            .......... .......... .......... .......... ..........
            .......... .......... .........K GEARESRXEE KVNQPKXEVE
            SKKXEATRLE KIKTDRKKAE EAXRKAAEED KVKEKPAEQP QPAPAPQPEK
            PAPAPKPEN. .......... ....PAEQPK AEKPADQQAE EDYARRSEEE
            YNRLTQQQPP KTEKPAQPST XK........ .......... ..........
            .......... .......... .......... ......
Rct123c     .......... .......... .......... .......... ..........
            .........I KEXDESXSED YLKEGLRAPL QSKLDTKKAK LSKLEELSDK
            IDELDAEIAK LEVQLKDAEG NNNVE..... A.YFKEGLEK TTAEKKAELE
            KAEADLKKAV DEPETPAPA. ......PQPA PAPEKPAE.. .........K
            PAPAPAP... .......... .......... .......... EKPAPAPEK.
            .......... ....PAPTPE .KPAPTPETP KTGWKQENGM WYFYNTDGSM
            ATGWLQNNGS WYYLNSNGAM ATGWHQNNGS WYYLNS
Rct129c     .......... .......... .......... .......... ..........
            .........L KEIDESDSED YLKEGLRAPL QSKLDTKKAK LSKLEELSDK
            IDELDAEIAK LEVQLKDAEG NNNVE..... A.YFKEGLEK TTAEKKAELE
            KAEADLKKAV DEPDTPAPA. ......PQPA PAPEKPAE.. .........K
            PAPAPAP... EKPAPAPE.. ....K.PAPA PEKPAP..AP EKPAPAPEK.
            .......... ....PAPAPE .KPAPAPEKP APAPKPETPE TRLETRKRY.
            .......... .......... .......... ......
```

FIG. 13L

```
Rct135c   .......... .......... .......... .......... ..........
          .........L KEIDESDSED YLKEGLRAPL QSKLDTKKAK LSKLEELSDK
          IDELDAEIAK LEVQLKDAEG NNNVE..... A.YFKEGLEK TTAEKKAELE
          KAEADLKKAV DEPETPAPA. ......PQPA PAPEKPAE.. .........K
          ..PAPAP... EKPAPAPE.. ....K.PAPA P......... EKPAPAPEK.
          .......... ....PAPAPE .KPAPTPETP KTGWKQENGM ..........
          .......... .......... .......... ......
RX1c      .......... .......... .......... .......... ..........
          .........L KEIDESESED YAKEGFRAPL QSKLDAKKAK LSKLEELSDK
          IDELDAEIAK LEDQLKAAEE NNNVE..... .DYFKEGLEK TIAAKKAELE
          KTEADLKKAV NEPEKPA... .....PAPET PAPEAPAE.. .......QPK
          PAPAPQP... ...APAPKPE K...PAEQPK PEKTDDQQAE EDYARRSEEE
          YNRLTQQQPP KAEKPAPA.. PKTGWKQENG MWYFYNTDGS M.........
          .......... .......... .......... ......
Bg6692c   .......... .......... .......... .......... ..........
          .......... .......... .......... .......... ..........
          .......... .......... ...GEQA... .GQYRAAAEG DLAAKQAELE
          KTEADLKKAV NEPEK..PA. .....PAPET PAPEAPAE.. .......QPK
          PAPAPQP... ...APAPKPE K...PAEQPK AEKTDDQQAE EDYARRSEEE
          YNRLTQQQPP KAEKPAPA.. PKPEQPAPA. .......... ..........
          .......... .......... .......... ......
```

FIG. 13M

```
Bg8838c   .......... .......... .......... .......... ..........
          .......... .......... .......... .......... ..........
          .......... ........PK NSKGEQA... .EQYRSAAGG DLAAKQVELE
          KTEADLKKAV NEPEK..PA. .....PAPET PAPEAPAE.. .......QPK
          PAPAPQP... ...APAPKPE K...PAEQPK AEKPADQQAE EDYDRRSEEE
          YNRLTQQQPP KAEKPAPA.. PQPEQPAPAP KS........ ..........
          .......... .......... .......... ......
Db16ac    .......... .......... .......... .......... ..........
          .........L KEIDESDSED YVKEGFRAPL QSELDAKQAK LSKLEELSDK
          IDELDAEIAK .LEKDVEDFK XSDGEQA... .GQYLAAAEE DLIAKKAELE
          QTEADLKKAV NEPGKPAPA. .....PAPET PAPEAPAE.. .......QPK
          PAPET.P... ...APAPKPE K...PAEQPK PEKPADQQAE EDYARRSEEE
          YNRLTQQQPA PAQKPEQP.. AKPEKPAEEP TQPEK..... ..........
          .......... .......... .......... ......
Db11c     .......... .......... .......... .......... ..........
          .......... .......... .......... .......... ..........
          ....DAEIAK .LEKNVEYFK KTDAEQT... .EQYLAAAEK DLADKKAELE
          KTEADLKKAV NEPEKPAEE. .....TPAPA PKPEQPAE.. .......QPK
          PAPAPQP... ...APAPKP. .......... .EKTDDQQAE EDYARRSEEE
          YNRLPQQQPP KAEKPAPA.. PKPEQPVP.. .......... ..........
          .......... .......... .......... ......
```

FIG. 13N

```
L820131c   .......... .......... .......... .......... ..........
           .......... .......... .......... .......... ..........
           .......... .......... .......... .......... ..........
           .......... .......... .........A EXPENPAP.. .......APK
           PAXAPQPLKP EEPAEQPKPE KPEEPAGQPE PEKPDDQQAG EDYARRSGGE
           YNRFPQQQPP KAEKPAPA.. PKPEQPVPAP KT........ ..........
           .......... .......... .......... ......
Bg11703c   .......... .......... .......... .....LLKKA KLAGAKSKAA
           TKKAELEPEL EKAEAELENL LSTLDPEGKT QDELDKEAAE ....AELNKK
           VEALPNQVSE LEEELSKLED NLKDAETNNV EDYIKEGLEE AIATKQAELE
           KT.......P KELDAALNEL G.PDGDEEET PPPEAPAE.. .......QPK
           PEK.PAEET. .......... ....PAPAPK PEKSADQQAE EDYARRSEEE
           YNRLTQQQPP KAEKPAPAPA PKPEQPAPAP KSR....... ..........
           .......... .......... .......... ......
Bg7817c    .......... .......... .......... ..GLATKKKL NLAEARIELL
           LKKLGLEPGL EKAGAGLGNL LSTLDPEGKT QDELDKEAAE ....AELNKK
           VEALPNQVAE LEEELSKLED NLKDAETNHV EDYIKEGLEE AIATKQAELE
           KT.......P KELDAALNEL G.PDGDEEET PAPEAPAE.. .......QPK
           PEK.PAEET. .......... ....PAPAPK PEKSADQQAE EDYARRSEEE
           YNRLTQQQPP KAEKPAPAPA PKPEQPAPAP K......... ..........
           .......... .......... .......... ......
```

FIG.130

```
Bg7561c   .......... .......... .......... .......... ..........
          .......... KKQKVNLENL LSTLDPGGKT QDELDKGAAE ....AELNKK
          VEALPNPVXE LEEELSPPED NLKDAETNHV EDYIKEGLEE AIATKQAELE
          ET.......P QEVDAALNDL V.PDGGEEET PAPAPQPD.. .......EPA
          PAPAPNAEQ. .......... ....PAPAPK PEKSADQQAE EDYARRSEGE
          YNRLTQQQPP KAEKPAPAPA PKPEQPAPAP N......... ..........
          .......... .......... .......... ......

Ef5668c   .....KEIAR LQSDLKDAEE NNVEDYIKEG LEQAITNKKA ELATTQQNID
          KT....QKDL EDAELELEKV LATLDPEGKT QDELDKEAAE ....AELNEK
          VEALQNQVAE LEEELSKLED NLKDAETNNV EDYIKEGLEE AIATKKAELE
          KT.......Q KELDAALNEL G.PDGDEEET PAPAPQPE.. .......KPA
          EEP....EN. .......... ....PAPAPK PEKSADQQAE EDYARRSEEE
          YNRLTQQQPP KAEK..PAPA PQPEQPAPAP KIE....... ..........
          .......... .......... .......... ......

Wu2c      .......... .......... .......... .......... ..........
          .........L KEIDESESED YAKEGFRAPL HSKLDAKKAK LSKLEELSDK
          IDELDAEIAK LEDQLKAVEE NNNVE..... .DYSTEGLEK TIAAKKTELE
          KTEADLKKAV NEPEKSAEEP SQPEKPAEEA PAPEQPTEPT ..........
          .....QPEKP AEETPAPKPE K...PAEQPN AEKTDDQQAE EDYARRSEEE
          YNRLTQQQPP KAEKPAPA.. PQPEQTSSLH .......... ..........
          .......... .......... .......... ......
```

FIG. 13P

Complete sequence for EF5668 pspA
Sequence Range: 1 to 1453

```
         10         20         30         40         50         60         70
          *          *          *          *          *          *          *
TTGACAAATA TTTACGGAGG AGGCTTATGC TTAATATAAG TATAGGCTAA AAATGATTAT CAGAAAAGAG

        80         90        100        110        120        130
         *          *          *          *          *          *
GTAAATTTAG ATG AAT AAG AAA AAA ATG ATT TTA ACA AGC CTA GCC AGC GTC GCT ATC TTA GGG
            M   N   K   K   K   M   I   L   T   S   L   A   S   V   A   I   L   G>

    140        150        160        170        180        190
      *          *          *          *          *          *
GCT GGT TTT GTT GCG TCT TCG CCT ACT TTT GTA AGA GCA GAA GAA GCT CCT GTA GCT AAC
 A   G   F   V   A   S   S   P   T   F   V   R   A   E   E   A   P   V   A   N>

    200        210        220        230        240        250
      *          *          *          *          *          *
CAG TCT AAA GCT GAG AAA GAC TAT GAT GCA GCA GTG AAA AAA TCT GAA GCT GCT AAG AAA
 Q   S   K   A   E   K   D   Y   D   A   A   V   K   K   S   E   A   A   K   K>

    260        270        280        290        300        310
      *          *          *          *          *          *
GAT TAC GAA ACG GCT AAA AAG AAA GCA GAA GAC GCT CAG AAG AAA TAT GAT GAG GAT CAG
 D   Y   E   T   A   K   K   K   A   E   D   A   Q   K   K   Y   D   E   D   Q>

    320        330        340        350        360        370
      *          *          *          *          *          *
AAG AAA ACT GAG GCA AAA GCG GAA AAA GAA AGA AAA GCT TCT GAA AAG ATA GCT GAG GCA
 K   K   T   E   A   K   A   E   K   E   R   K   A   S   E   K   I   A   E   A>

    380        390        400        410        420        430
      *          *          *          *          *          *
```

FIG. 13Q

```
ACA AAA GAA GTT CAA CAA GCG TAC CTA GCT TAT CTA CAA GCT AGC AAC GAA AGT CAG AGA
 T   K   E   V   Q   Q   A   Y   L   A   Y   L   Q   A   S   N   E   S   Q   R>

     440         450         460         470         480         490
      *           *           *           *           *           *
AAA GAG GCA GAT AAG AAG ATA AAA GAA GCT ACG CAC GCA AAG ATG AGG CGG ACG TGC AAT
 K   E   A   D   K   K   I   K   E   A   T   H   A   K   M   R   R   T   C   N>

     500         510         520         530         540         550
      *           *           *           *           *           *
TTG ACT ATC GAA TTC GAA CAA CAA TTG TAC TTC CTG AAC CAA GTG AGT TAC CTG AGA CTA
 L   T   I   E   F   E   Q   Q   L   Y   F   L   N   Q   V   S   Y   L   R   L>

     560         570         580         590         600         610
      *           *           *           *           *           *
AGA AAA AAG CAG AAG AGG CAA CAA AAG AAG CAG AAG TAT CTA AGA AAA AAT CTG AAG AGG
 R   K   K   Q   K   R   Q   Q   K   K   Q   K   Y   L   R   K   N   L   K   R>

     620         630         640         650         660         670
      *           *           *           *           *           *
CAG CTA AAG AGG TAT AAG TAT AGA AAA ATA AAA TAC TTG AAC AAG ATG CTG AAA ACG AAA
 Q   L   K   R   Y   K   Y   R   K   I   K   Y   L   N   K   M   L   K   T   K>

     680         690         700         710         720         730
      *           *           *           *           *           *
AGA AAA TTG ACG TAC TTC AAA ACA AAG TCG CTG ATT TAT AAA AAG GAA TTG CTC TCC ATC
 R   K   L   T   Y   F   K   T   K   S   L   I   Y   K   K   E   L   L   S   I>

     740         750         760         770         780         790
      *           *           *           *           *           *
AAA ACA GTC GCT GAA TTA AAT AAA GAA ATT GCT AGA CTT CAA AGC GAT TTA AAA GAT GCT
 K   T>
       V   A   E   L   N   K   E   I   A   R   L   Q   S   D   L   K   D   A>

     800         810         820         830         840         850
      *           *           *           *           *           *
```

FIG.13R

```
GAA GAA AAT AAT GTA GAA GAC TAC ATT AAA GAA GGT TTA GAG CAA GCT ATC ACT AAT AAA
 E   E   N   N   V   E   D   Y   I   K   E   G   L   E   Q   A   I   T   N   K>

    860       870       880       890       900       910
      *         *         *         *         *         *

AAA GCT GAA TTA GCT ACA ACT CAA CAA AAC ATA GAT AAA ACT CAA AAA GAT TTA GAG GAT
 K   A   E   L   A   T   T   Q   Q   N   I   D   K   T   Q   K   D   L   E   D>

    920       930       940       950       960       970
      *         *         *         *         *         *

GCT GAA TTA GAA CTT GAA AAA GTA TTA GCT ACA TTA GAC CCT GAA GGT AAA ACT CAA GAT
 A   E   L   E   L   E   K   V   L   A   T   L   D   P   E   G   K   T   Q   D>

    980       990      1000      1010      1020      1030
      *         *         *         *         *         *

GAA TTA GAT AAA GAA GCT GCT GAA GCT GAG TTG AAT GAA AAA GTT GAA GCT CTT CAA AAC
 E   L   D   K   E   A   A   E   A   E   L   N   E   K   V   E   A   L   Q   N>

   1040      1050      1060      1070      1080      1090
      *         *         *         *         *         *

CAA GTT GCT GAA TTA GAA GAA GAA CTT TCA AAA CTT GAA GAT AAT CTT AAA GAT GCT GAA
 Q   V   A   E   L   E   E   E   L   S   K   L   E   D   N   L   K   D   A   E>

   1100      1110      1120      1130      1140      1150
      *         *         *         *         *         *
```

FIG. 13S

```
ACA AAC AAC GTT GAA GAC TAC ATT AAA GAA GGT TTA GAA GAA GCT ATC GCG ACT AAA AAA
 T   N   N   V   E   D   Y   I   K   E   G   L   E   E   A   I   A   T   K   K>

   1160          1170         1180         1190          1200         1210
      *             *            *            *             *            *
GCT GAA TTG GAA AAA ACT CAA AAA GAA TTA GAT CCA CCT CTT AAT GAG TTA GGC CCT GAT
 A   E   L   E   K   T   Q   K   E   L   D   A   A   L   N   E   L   G   P   D>

   1220          1230         1240         1250          1260         1270
      *             *            *            *             *            *
GGA GAT GAA GAA GAG ACT CCA GCG CCG GCT CCT CAA CCA GAA AAA CCA GCT GAA GAG CCT
 G   D   E   E   E   T   P   A   P   A   P   Q   P   E   K   P   A   E   E   P>

   1280          1290         1300         1310          1320         1330
      *             *            *            *             *            *
GAG AAT CCA GCT CCA GCA CCA AAA CCA GAG AAG TCA GCA GAT CAA CAA GCT GAA GAA GAC
 E   N   P   A   P   A   P   K   P   E   K   S   A   D   Q   Q   A   E   E   D>

   1340          1350         1360         1370          1380         1390
      *             *            *            *             *            *
TAT GCT CGT AGA TCA GAA GAA GAA TAT AAT CGC TTG ACC CAA CAG CAA CCG CCA AAA GCA
 Y   A   R   R   S   E   E   E   Y   N   R   L   T   Q   Q   Q   P   P   K   A>

   1400          1410         1420         1430          1440         1450
      *             *            *            *             *            *
GAA AAA CCA GCT CCT GCA CCA CAA CCA GAG CAA CCA GCT CCT GCA CCA AAA ATA GAG GC
 E   K   P   A   P   A   P   Q   P   E   Q   P   A   P   A   P   K   I   E   A>
```

FIG. 13T

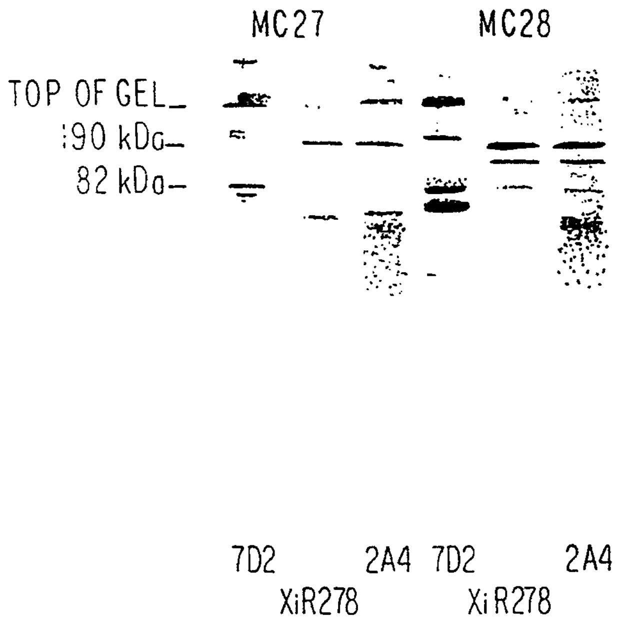

MC27
MC28
TOP OF GEL
190 kDa
82 kDa
7D2
2A4
7D2
2A4
XiR278
XiR278

FIG. 14

FIG. 15A

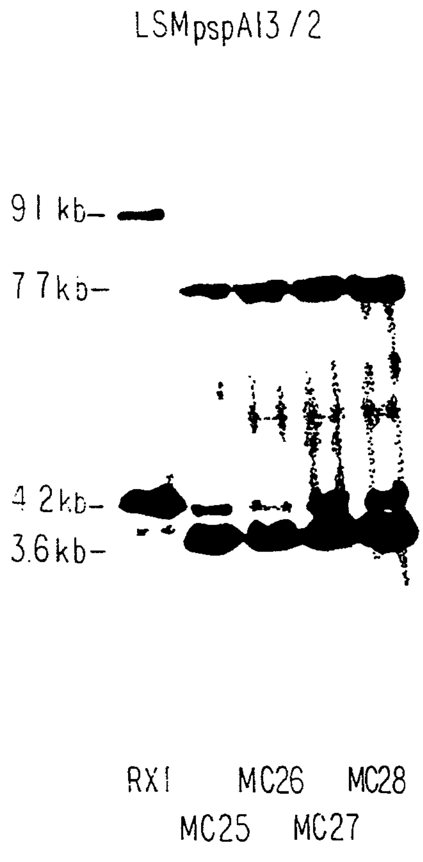
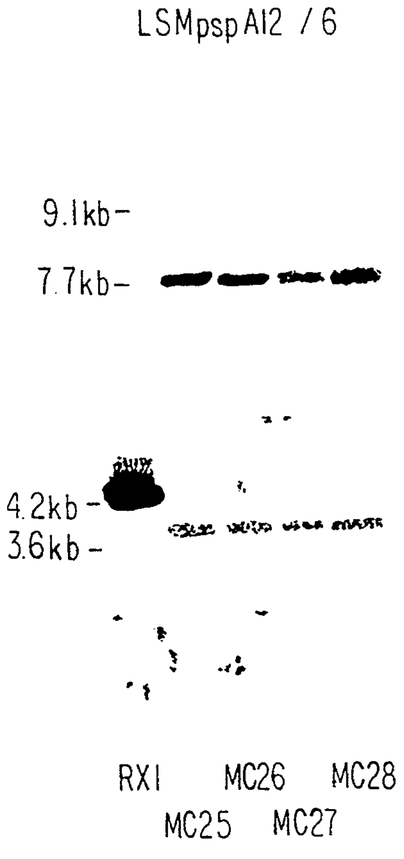


FIG. 15B



```
Primer LSM13:  gcaagcttatgatatagaaatttgtaac
Primer LSM2:  gcgcgtcgacggcttaaacccattcaccattgg

Probe LSMpspA13/2 (from RX1 sequence):
aagcttatga tatagaaatt tgtaacaaaa atgtaatata aaacacttga
caaatattta cggaggaggc ttatacttaa tataagtata gtctgaaaat
gactatcaga aaagaggtaa atttagatga ataagaaaaa aatgatttta
acaagtctag ccagcgtcgc tatcttaggg gctggttttg ttgcgtctca
gcctactgtt gtaagagcag aagaatctcc cgtagccagt cagtctaaag
ctgagaaaga ctatgatgca gcgaagaaag atgctaagaa tgcgaaaaaa
gcagtagaag atgctcaaaa ggctttagat gatgcaaaag ctgctcagaa
aaaatatgac gaggatcaga agaaaactga ggagaaagcc gcgctagaaa
aagcagcgtc tgaagagatg gataaggcag tggcagcagt tcaacaagcg
tatctagcct atcaacaagc tacagacaaa gccgcaaaag acgcagcaga
taagatgata gatgaagcta agaaacgcga agaagaggca aaaactaaat
ttaatactgt tcgagcaatg gtagttcctg agccagagca gttggctgag
actaagaaaa aatcagaaga agctaaacaa aaagcaccag aacttactaa
aaaactagaa gaagctaaag caaaattaga agaggctgag aaaaaagcta
ctgaagccaa acaaaaagtg gatgctgaag aagtcgctcc tcaagctaaa
atcgctgaat tggaaaatca agttcataga ctagaacaag agctcaaaga
gattgatgag tctgaatcag aagattatgc taaagaaggt ttccgtgctc
ctcttcaatc taaattggat gccaaaaaag ctaaactatc aaaacttgaa
```

FIG. 15C

```
gagttaagtg ataagattga tgagttagac gctgaaattg caaaacttga
agatcaactt aaagctgctg aagaaaacaa taatgtagaa gactacttta
aagaaggttt agagaaaact attgctgcta aaaaagctga attagaaaaa
actgaagctg accttaagaa agcagttaat gagccagaaa aaccagctcc
agctccagaa actccagccc cagaagcacc agctgaacaa ccaaaaccag
cgccggctcc tcaaccagct cccgcaccaa aaccagagaa gccagctgaa
caaccaaaac cagaaaaaac agatgatcaa caagctgaag aagactatgc
tcgtagatca gaagaagaat ataatcgctt gactcaacag caaccgccaa
aagctgaaaa accagctcct gcaccaaaaa caggctggaa acaagaaaac
ggtatgtggt acttctacaa tactgatggt tcaatggcga caggatggct
ccaaaacaac ggttcatggt actacctcaa cagcaatggt gctatggcta
caggttggct ccaatacaat ggttcatggt attacctcaa cgctaacggc
gctatggcaa caggttgggc taaagtcaac ggttcatggt actacctcaa
cgctaatggt gctatggcta caggttggct ccaatacaac ggttcatggt
attacctcaa cgctaacggc gctatggcaa caggttgggc taaagtcaac
ggttcatggt actacctcaa cgctaatggt gctatggcta caggttggct
ccaatacaac ggttcatggt actacctcaa cgctaacggt gctatggcta
caggttgggc taaagtcaac ggttcatggt actacctcaa cgctaatggt
gctatggcaa caggttgggt gaaagatgga gatacctggt actatcttga
agcatcaggt gctatgaaag caagccaatg gttcaaagta tcagataaat
ggtactatgt caatggttta ggtgcccttg cagtcaacac aactgtagat
ggctataaag tcaatgccaa tggtgaatgg gtttaagccg
```

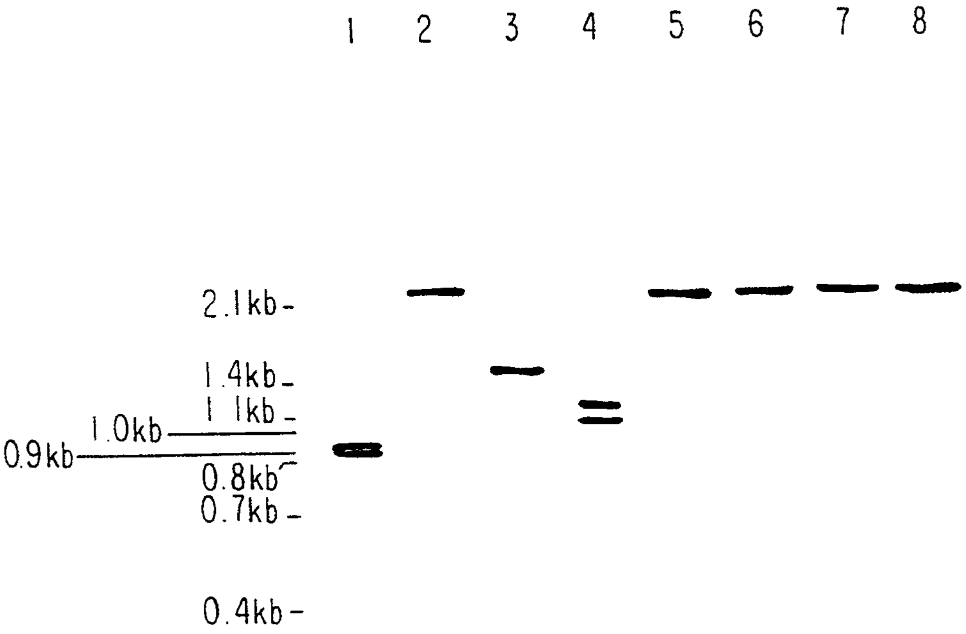
FIG. 15D

```
Primer LSM12:  ccggatccagcgtcgctatcttaggggctggtt
Primer LSM6:  ctgagtcgactggagtttctggagctggagc

Probe LSMpspA12/6 (from RX1 sequence):
ccagcgtcgc tatcttaggg gctggttttg ttgcgtctca gcctactgtt
gtaagagcag aagaatctcc cgtagccagt cagtctaaag ctgagaaaga
ctatgatgca gcgaagaaag atgctaagaa tgcgaaaaaa gcagtagaag
atgctcaaaa ggctttagat gatgcaaaag ctgctcagaa aaaatatgac
gaggatcaga agaaaactga ggagaaagcc gcgctagaaa aagcagcgtc
tgaagagatg gataaggcag tggcagcagt tcaacaagcg tatctagcct
atcaacaagc tacagacaaa gccgcaaaag acgcagcaga taagatgata
gatgaagcta agaaacgcga agaagaggca aaaactaaat ttaatactgt
tcgagcaatg gtagttcctg agccagagca gttggctgag actaagaaaa
aatcagaaga agctaaacaa aaagcaccag aacttactaa aaaactagaa
gaagctaaag caaaattaga agaggctgag aaaaaagcta ctgaagccaa
acaaaaagtg gatgctgaag aagtcgctcc tcaagctaaa atcgctgaat
tggaaaatca agttcataga ctagaacaag agctcaaaga gattgatgag
tctgaatcag aagattatgc taaagaaggt ttccgtgctc ctcttcaatc
taaattggat gccaaaaaag ctaaactatc aaaacttgaa gagttaagtg
ataagattga tgagttagac gctgaaattg caaaacttga agatcaactt
aaagctgctg aagaaaacaa taatgtagaa gactacttta aagaaggttt
agagaaaact attgctgcta aaaaagctga attagaaaaa actgaagctg
accttaagaa agcagttaat gagccagaaa aaccagctcc agctccagaa
actccag
```

FIG. 15E

FIG. 16



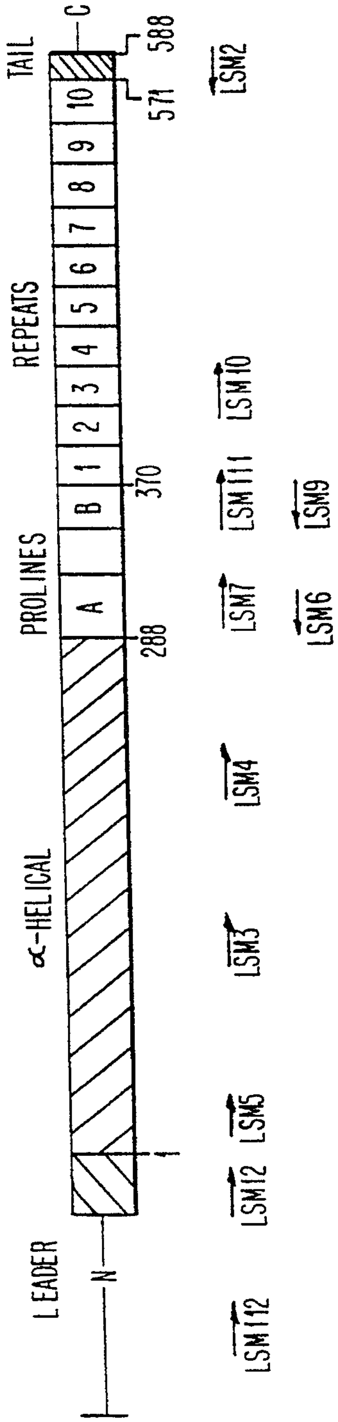

LEADER
N
α-HELICAL
PROLINES
A
B
288
370
REPEATS
1
2
3
4
5
6
7
8
9
10
TAIL
571
588
C
LSM112
LSM12
LSM5
LSM3
LSM4
LSM7
LSM6
LSM111
LSM9
LSM10
LSM2

FIG. 17

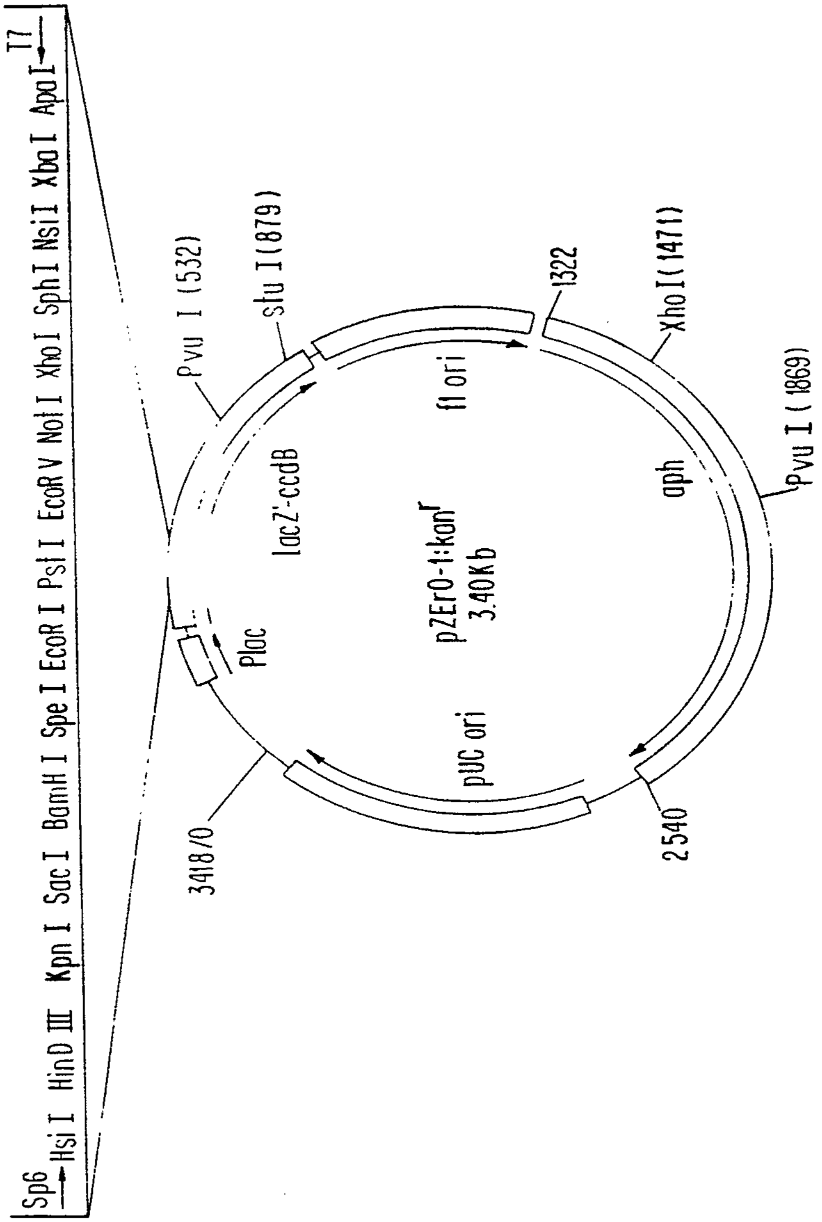

Sp6
Hsi I
HinD III
Kpn I
Sac I
BamH I
Spe I
EcoR I
Pst I
EcoR V
Not I
Xho I
Sph I
Nsi I
Xba I
Apa I
T7
Pvu I (532)
stu I (879)
1322
XhoI (1471)
Pvu I (1869)
2540
3418/0
lacZ'-ccdB
f1 ori
aph
Plac
pUC ori
pZErO-1:kan^r
3.40Kb

FIG. 18

SKH2	5' CAT ACC gTT TTC TTg TTT CCA gCC -3'
LSM13	5' gCA AgC TTA TgA TAT AgA AAT TTg TAA C -3'
N192	5' ggAAggCCATATgCTCAAAgAgATTgATgAgTCT -3'
C588	5' CCAAggATCCTTAAACCCATTCACCATTggC -3'

FIG. 19

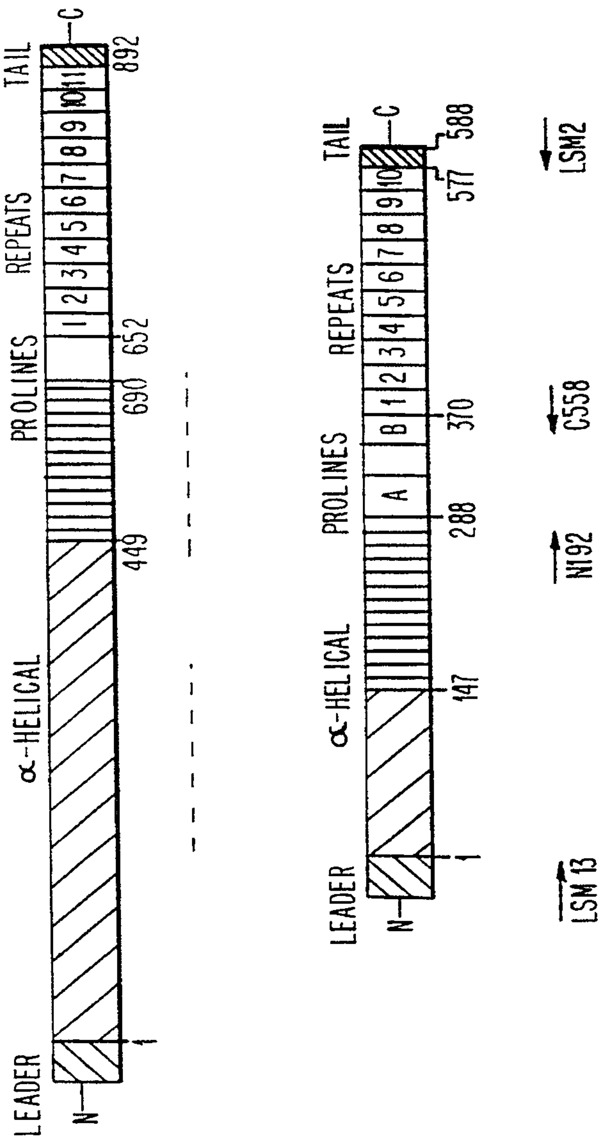

LEADER
N
1
α-HELICAL
449
PROLINES
690
652
REPEATS
1
2
3
4
5
6
7
8
9
10
11
TAIL
892
C
LEADER
N
1
α-HELICAL
147
PROLINES
A
288
B
370
REPEATS
1
2
3
4
5
6
7
8
9
10
577
TAIL
588
C
LSM13
N192
C558
LSM2

FIG. 20

```
AAGCTTATGC TTGTCAATAA TCACAAATAT GTAGATCATA TCTTGTTTAG GACAGTAAAA CATCCTAATT ACTTTTTAAA   80

TATTTTACCT GAGTTGATTG GCTTGACCTT GTTGAGTCAT GCCTATATGA CTTTTGTTTT AGTTTTTCCA GTTTATGCAG  160

TTATTTTGTA TCGACGAATA GCTGAAGAGG AAAAGTTATT ACATGAAGTT ATAATCCCAA ATGGAAGCAT AAAGAGATAA  240

ATACAAAATT CGATTTATAT ACAGTTCATA TTGAAGTGAT ATAGTAAGGT TAAAGAAAAA ATAtagaagg aAATAAACAT  320
                                                                                  Met> -37

GTTTGCATCA AAAAGCGAAA GAAAAGTACA TTATTCAATT CGTAAATTTA GTATTGGAGT AGCTAGTGTA GCTGTTGCCA  400
 PheAlaSer LysSerGlu ArgLysValHis TyrSerIle ArgLysPhe SerIleGlyVal AlaSerVal AlaValAla>  -11

 GCTTGTTCTT AGGAGGAGTA GTCCATGCAG AAGGGGTTAG AAGTGGGAAT AACCTCACGG TTACATCTAG TGGGCAAGAT 480
SerLeuPheLeu GlyGlyVal ValHisAla GluGlyValArg SerGlyAsn AsnLeuThr ValThrSerSer GlyGlnAsp> 17

ATATCGAAGA AGTATGCTGA TGAAGTCGAG TCGCATCTAG AAAGTATATT GAAGGATGTC AAAAAAAATT TGAAAAAAGT  560
IleSerLys LysTyrAlaAsp GluValGlu SerHisLeu GluSerIleLeu LysAspVal LysLysAsn LeuLysLysVal> 44

TCAACATACC CAAAATGTCG GCTTAATTAC AAAGTTGAGC GAAATTAAAA AGAAGTATTT GTATGACTTA AAAGTTAATG  640
 GlnHisThr GlnAsnVal GlyLeuIleThr LysLeuSer GluIleLys LysLysTyrLeu TyrAspLeu LysValAsn>  70

 TTTTATCGGA AGCTGAGTTG ACGTCAAAAA CAAAAGAAAC AAAAGAAAAG TTAACCGCAA CTTTTGAGCA GTTTAAAAAA 720
ValLeuSerGlu AlaGluLeu ThrSerLys ThrLysGluThr LysGluLys LeuThrAla ThrPheGluGln PheLysLys> 97

GATACATTAC CAACAGAACC AGAAAAAAAG GTAGCAGAAG CTCAGAAGAA GGTTGAAGAA GCTAAGAAAA AAGCCGAGGA  800
AspThrLeu ProThrGluPro GluLysLys ValAlaGlu AlaGlnLysLys ValGluGlu AlaLysLys LysAlaGluAsp> 124
```

FIG. 21A

```
TCAAAAAGAA AAAGATCGCC GTAACTACCC AACCATTACT TACAAAACGC TTGAACTTGA AATTGCTGAG TCCGATGTGG  880
 GlnLysGlu LysAspArg ArgAsnTyrPro ThrIleThr TyrLysThr LeuGluLeuGlu IleAlaGlu SerAspVal>  150

 AAGTTAAAAA AGCGGAGCTT GAACTAGTAA AAGTGAAAGC TAAGGAATCT CAAGACGAGG AAAAAATTAA GCAAGCAGAA  960
GluValLysLys AlaGluLeu GluLeuVal LysValLysAla LysGluSer GlnAspGlu GluLysIleLys GlnAlaGlu> 177

GCGGAAGTTG AGAGTAAACA AGCTGAGGCT ACAAGGTTAA AAAAAATCAA GACAGATCGT GAAGAAGCTA AACGAAAAGC  1040
AlaGluVal GluSerLysGln AlaGluAla ThrArgLeu LysLysIleLys ThrAspArg GluGluAla LysArgLysAla> 204

AGATGCTAAG TTGAAGGAAG CTGTTGAAAA GAATGTAGCG ACTTCAGAGC AAGATAAACC AAAGAGGCGG GCAAAACGAG  1120
 AspAlaLys LeuLysGlu AlaValGluLys AsnValAla ThrSerGlu GlnAspLysPro LysArgArg AlaLysArg>  230

 GAGTTTCTGG AGAGCTAGCA ACACCTGATA AAAAAGAAAA TGATGCGAAG TCTTCAGATT CTAGCGTAGG TGAAGAAACT  1200
GlyValSerGly GluLeuAla ThrProAsp LysLysGluAsn AspAlaLys SerSerAsp SerSerValGly GluGluThr> 257

CTTCCAAGCC CATCCCTTAA TATGGCAAAT GAAAGTCAGA CAGAACATAG GAAAGATGTC GATGAATATA TAAAAAAAAT  1280

LeuProSer ProSerLeuAsn MetAlaAsn GluSerGln ThrGluHisArg LysAspVal AspGluTyr IleLysLysMet> 284

GTTGAGTGAG ATCCAATTAG ATAGAAGAAA ACATACCCAA AATGTCAACT TAAACATAAA GTTGAGCGCA ATTAAAACGA  1360
 LeuSerGlu IleGlnLeu AspArgArgLys HisThrGln AsnValAsn LeuAsnIleLys LeuSerAla IleLysThr>  310

 AGTATTTGTA TGAATTAAGT GTTTTAAAAG AGAACTCGAA AAAAGAAGAG TTGACGTCAA AAACCAAAGC AGAGTTAACC  1440
LysTyrLeuTyr GluLeuSer ValLeuLys GluAsnSerLys LysGluGlu LeuThrSer LysThrLysAla GluLeuThr> 337

GCAGCTTTTG AGCAGTTTAA AAAAGATACA TTGAAACCAG AAAAAAAGGT AGCAGAAGCT GAGAAGAAGG TTGAAGAAGC  1520
AlaAlaPhe GluGlnPheLys LysAspThr LeuLysPro GluLysLysVal AlaGluAla GluLysLys ValGluGluAla> 364
```

FIG. 21B

```
TAAGAAAAAA GCCAAGGATC AAAAAGAAGA AGATCGCCGT AACTACCCAA CCAATACTTA CAAAACGCTT GAACTTGAAA  1600
 LysLysLys AlaLysAsp GlnLysGluGlu AspArgArg AsnTyrPro ThrAsnThrTyr LysThrLeu GluLeuGlu>  390

 TTGCTGAGTC CGATGTGAAA GTTAAAGAAG CGGAGCTTGA ACTAGTAAAA GAGGAAGCTA ACGAATCTCG AAACGAGGAA 1680
IleAlaGluSer AspValLys ValLysGlu AlaGluLeuGlu LeuValLys GluGluAla AsnGluSerArg AsnGluGlu> 417

AAAATTAAGC AAGCAAAAGA GAAAGTTGAG AGTAAAAAAG CTGAGGCTAC AAGGTTAGAA AAAATCAAGA CAGATCGTAA  1760
LysIleLys GlnAlaLysGlu LysValGlu SerLysLys AlaGluAlaThr ArgLeuGlu LysIleLys ThrAspArgLys> 444

AAAAGCAGAA GAAGAAGCTA AACGAAAAGC AGAAGAATCT GAGAAAAAAG CTGCTGAAGC CAAACAAAAA GTGGATGCTG  1840
 LysAlaGlu GluGluAla LysArgLysAla GluGluSer GluLysLys AlaAlaGluAla LysGlnLys ValAspAla>  470

 AAGAATATGC TCTTGAAGCT AAAATCGCTG AGTTGGAATA TGAAGTTCAG AGACTAGAAA AAGAGCTCAA AGAGATTGAT 1920
GluGluTyrAla LeuGluAla LysIleAla GluLeuGluTyr GluValGln ArgLeuGlu LysGluLeuLys GluIleAsp> 497

GAGTCTGACT CAGAAGATTA TCTTAAAGAA GGCCTCCGTG CTCCTCTTCA ATCTAAATTG GATACCAAAA AAGCTAAACT  200
GluSerAsp SerGluAspTyr LeuLysGlu GlyLeuArg AlaProLeuGln SerLysLeu AspThrLys LysAlaLysLeu> 524

ATCAAAACTT GAAGAGTTGA GTGATAAGAT TGATGAGTTA GACGCTGAAA TTGCAAAACT TGAAGTTCAA CTTAAAGATG  2080
 SerLysLeu GluGluLeu SerAspLysIle AspGluLeu AspAlaGlu IleAlaLysLeu GluValGln LeuLysAsp>  550

 CTGAAGGAAA CAATAATGTA GAAGCCTACT TTAAAGAAGG TTTAGAGAAA ACTACTGCTG AGAAAAAAGC TGAATTAGAA 2160
AlaGluGlyAsn AsnAsnVal GluAlaTyr PheLysGluGly LeuGluLys ThrThrAla GluLysLysAla GluLeuGlu> 577

AAAGCTGAAG CTGACCTTAA GAAAGCAGTT GATGAGCCAG AAACTCCAGC TCCGGCTCCT CAACCAGCTC CAGCTCCAGA  2240
LysAlaGlu AlaAspLeuLys LysAlaVal AspGluPro GluThrProAla ProAlaPro GlnProAla ProAlaProGlu> 604
```

FIG. 21C

```
AAAACCAGCT GAAAAACCAG CTCCAGCTCC AGAAAAACCA GCTCCAGCTC CAGAAAAACC AGCTCCAGCT CCAGAAAAAC  2320
 LysProAla GluLysPro AlaProAlaPro GluLysPro AlaProAla ProGluLysPro AlaProAla ProGluLys>  630

 CAGCTCCAGC TCCAGAAAAA CCAGCTCCAG CTCCAGAAAA ACCAGCTCCA ACTCCAGAAA CTCCAAAAAC AGGCTGGAAA  2400
ProAlaProAla ProGluLys ProAlaPro AlaProGluLys ProAlaPro ThrProGlu ThrProLysThr GlyTrpLys> 657
CAAGAAAACG GTATGTGGTA CTTCTACAAT ACTGATGGTT CAATGGCAAC AGGCTGGCTC CAAAACAATG GCTCATGGTA  2480
GlnGluAsn GlyMetTrpTyr PheTyrAsn ThrAspGly SerMetAlaThr GlyTrpLeu GlnAsnAsn GlySerTrpTyr> 684

CTACCTCAAC AGCAATGGCG CTATGGCGAC AGGATGGCTC CAAAACAATG GCTCATGGTA CTACCTCAAC AGCAATGGCG  2560
 TyrLeuAsn SerAsnGly AlaMetAlaThr GlyTrpLeu GlnAsnAsn GlySerTrpTyr TyrLeuAsn SerAsnGly>  710

 CTATGGCGAC AGGATGGCTC CAATACAATG GTTCATGGTA CTACCTCAAC GCTAATGGTG ATATGGCGAC AGGATGGCTC  2640
AlaMetAlaThr GlyTrpLeu GlnTyrAsn GlySerTrpTyr TyrLeuAsn AlaAsnGly AspMetAlaThr GlyTrpLeu> 737

CAATACAATG GTTCATGGTA CTACCTCAAC GCTAATGGTG ATATGGCGAC AGGATGGTTC CAATACAATG GTTCATGGTA  2720
GlnTyrAsn GlySerTrpTyr TyrLeuAsn AlaAsnGly AspMetAlaThr GlyTrpPhe GlnTyrAsn GlySerTrpTyr> 764

CTACCTCAAC GCTAATGGTG ATATGGCGAC AGGATGGTTC CAATACAATG GTTCATGGTA CTACCTCAAC GCTAATGGTG  2800
 TyrLeuAsn AlaAsnGly AspMetAlaThr GlyTrpPhe GlnTyrAsn GlySerTrpTyr TyrLeuAsn AlaAsnGly>  790

 ATATGGCGAC AGGATGGCTC CAATACAATG GTTCATGGTA CTACCTAAAC AGCAATGGTG CTATGGTAAC AGGATGGCTC  2880
AspMetAlaThr GlyTrpLeu GlnTyrAsn GlySerTrpTyr TyrLeuAsn SerAsnGly AlaMetValThr GlyTrpLeu> 817

CAAAACAATG GCTCATGGTA CTACCTAAAC GCTAACGGTT CAATGGCAAC AGATTGGGTG AAAGATGGAG ATACCTGGTA  2960
GlnAsnAsn GlySerTrpTyr TyrLeuAsn AlaAsnGly SerMetAlaThr AspTrpVal LysAspGly AspThrTrpTyr> 844
```

FIG. 21D

```
CTATCTTGAA GCATCAGGTG CTATGAAAGC AAGCCAATGG TTCAAAGTAT CAGATAAATG GTACTATGTC AATGGCTCAG  3040
 TyrLeuGlu AlaSerGly AlaMetLysAla SerGlnTrp PheLysVal SerAspLysTrp TyrTyrVal AsnGlySer>  870

GTGCCCTTGC AGTCAACACA ACTGTAGATA GCTATAGAGT CAATGCCAAT GGTGAATGGG TAAACTAAAC TTAATATAAC
GlyAlaLeuAla ValAsnThr ThrValAsp SerTyrArgVal AsnAlaAsn GlyGluTrp ValAsn>               892

TAGTTAATAC TGACTTCCTG TAAGAACTCT TTAAAGTATT CCCTACAAAT ACCATATCCT TTCAGTAGAT AATATACCCT  3200

TGTAGGAAGT TTAGATTAAA AAATAACTCT GTAATCTCTA GCCGGATTTA TAGCGCTAGA GACTACGGAG TTTTTTTGAT  3280

GAGGAAAGAA TGGCGGCATT CAAGAGACTC TTTAAGAGAG TTACGGGTTT TAAACTATTA AGCTTTCTCC AATTGCAAGA  3360

GGGCTTCAAT CTCTGCTAGG TGCTAGCTTG CGAAATGGCT CCCACGGAGT TTGGCRGCGC CAGATGTTCC ACGGAGGTAG  3440

TGAGGAGCGA GGCCGCGGAA TTC
```

FIG. 2/E

```
249 SDSSVGEETLPSPSLNMANESQTEHRKDVDEYIKKMLSEIQLDRRKHTQN 298
    .:|.|:.:. :....: |.....: :|.|::  .| |.: . ..:|..::
  1 EESPVASQSKAEKDYDAAKKDAKNAKKAVED.AQKALDDAKAAQKKYDED 49
             .         .         .         .         .
299 VNLNIKLSAIKTKYLYELSVLKENSKKEELTSKTKAELTAAFEQFKKDTL 348
     . . . .|:            |.. .||: .|. |.: .|: .:...|
 50 QKKTEEKAAL............EKAASEEM.DKAVAAVQQAYLAYQQATD 86
             .         .         .         .         .
349 KPEKKVAEAEKKVEEAKKKAKDQKEEDRRNYPTNTYKTLELEIAESDVKV 398
    |:.|  ..|:|.::||||:..:.|..       || :.: :
 87 KAAK..DAADKMIDEAKKREEEAKTK......FNTVRAMVV......... 119
             .         .         .         .         .
399 KEAELELVKEEANESRNEEKIKQAKEKVESKKAEATRLEKIKTDRKKAEE 448
                   .:.|.: :.|.| |. |..|. |       .|  |
120 ...............PEPEQLAETKKKSEEAKQKAPEL.......TKKLE 147
             .         .         .         .         .
449 EAKRKAEESEKKAAEAKQKVDAEEYALEAKIAELEYEVQRLEKELKEIDE 498
    ||| | ||.||||.|||||||||| | :||||||| :|:|||.|||||||
148 EAKAKLEEAEKKATEAKQKVDAEEVAPQAKIAELENQVHRLEQELKEIDE 197
```

FIG. 22A

```
              .         .         .         .         .
499 SDSEDYLKEGLRAPLQSKLDTKKAKLSKLEELSDKIDELDAEIAKLEVQL 548
    |:|||| |||:|||||||||.|||||||||||||||||||||||||| ||
198 SESEDYAKEGFRAPLQSKLDAKKAKLSKLEELSDKIDELDAEIAKLEDQL 247

              .         .         .         .         .
549 KDAEGNNNVEAYFKEGLEKTTAEKKAELEKAEADLKKAVDEPETPAPAPQ 598
    |.||:|||||.|||||||||.|.|||||||.||||||||:|||.|||||:
248 KAAEENNNVEDYFKEGLEKTIAAKKAELEKTEADLKKAVNEPEKPAPAPE 297

              .         .         .         .         .
599 PAPAPEKPAE..KPAPAPEK.PAPAPEKPA..PAPEKPAPA......... 634
     .|||| |||  ||||||:. ||| |||||  | |||....
298 .TPAPEAPAEQPKPAPAPQPAPAPKPEKPAEQPKPEKTDDQQAEEDYARR 346

              .         .         .         .         .
635 ........PEKPAPAPEKPAPTPETPKTGWKQENGMWYFYNTDGSMATGW 676
            .:..:| :|||||   .||||||||||||||||||||
347 SEEEYNRLTQQQPPKAEKPAP...APKTGWKQENGMWYFYNTDGS..... 388

              .         .         .         .         .
677 LQNNGSWYYLNSNGAMATGWLQNNGSWYYLNSNGAMATGWLQYNGSWYYL 726
                   |||||||||||||||||||||||||||||||||||
389 ...............MATGWLQNNGSWYYLNSNGAMATGWLQYNGSWYYL 423
```

FIG. 22B

```
             .         .         .         .         .
727 NANGDMATGWLQYNGSWYYLNANGDMATGWFQYNGSWYYLNANGDMATGW 776
    ||||.||||| . |||||||||||.|||||:||||||||||||||.|||||
424 NANGAMATGWAKVNGSWYYLNANGAMATGWLQYNGSWYYLNANGAMATGW 473

             .         .         .         .         .
777 FQYNGSWYYLNANGDMATGWLQYNGSWYYLNSNGAMVTGWLQNNGSWYYL 826
     . |||||||||||.||||||||||||||||.||||.||| . |||||||
474 AKVNGSWYYLNANGAMATGWLQYNGSWYYLNANGAMATGWAKVNGSWYYL 523

             .         .         .         .         .
827 NANGSMATDWVKDGDTWYYLEASGAMKASQWFKVSDKWYYVNGSGALAVN 876
    ||||.|||:|||||||||||||||||||||||||||||||||| ||||||
524 NANGAMATGWVKDGDTWYYLEASGAMKASQWFKVSDKWYYVNGLGALAVN 573

             .
877 TTVDSYRVNANGEWV 891
    ||||:|:||||||||
574 TTVDGYKVNANGEWV 588
```

FIG. 22C

	a	b	c	d	e	f	g	
1								Glu Gly Val Arg Ser Gly Asn Asn Leu Thr
11								Val Thr Ser Ser Gly
16		Gln	Asp	Ile	Ser	Lys	Lys	
22	Tyr	Ala	Asp	Glu	Val	Glu	Ser	
29			His	Leu	Glu	Ser	Ile	
34	Leu	Lys	Asp	Val	Lys	Lys	Asn	
41	Leu	Lys	Lys					
44	Val	Gln	His	Thr	Gln	Asn	Val	
51			Gly	Leu	Ile	Thr	Lys	
56	Leu	Ser	Glu	Ile	Lys	Lys	Lys	
63							Tyr	
64	Leu	Tyr	Asp	Leu	Lys			
69	Val	Asn	Val	Leu	Ser	Glu	Ala	
76			Glu	Leu	Thr	Ser	Lys	
81				Thr	Lys	Glu	Thr	
85	Lys	Glu	Lys	Leu	Thr	Ala	Thr	
92	Phe	Glu	Gln	Phe	Lys	Lys	Asp	
99								Thr Leu Pro Thr Glu Pro
105					Glu	Lys	Lys	
108	Val	Ala	Glu	Ala	Gln	Lys	Lys	
115	Val	Glu	Glu	Ala	Lys	Lys	Lys	

FIG. 23A

```
122                     Ala Glu Asp Gln
126  Lys Glu Lys Asp Arg Arg Asn
133  Tyr Pro Thr Ile Thr
138  Tyr Lys Thr Leu Glu Leu Glu
145  Ile Ala Glu Ser Asp Val Glu
152  Val Lys Lys Ala Glu Leu Glu
159  Leu Val Lys Val Lys Ala Lys
166  Glu Ser Gln Asp Glu Glu Lys
173  Ile Lys Gln Ala Glu Ala Glu
180  Val Glu Ser Lys Gln Ala Glu
137              Ala Thr Arg
190  Leu Lys Lys Ile Lys Thr Asp
197  Arg Glu Glu Ala Lys Arg Lys
204      Ala Asp Ala Lys Leu Lys
210      Glu Ala Val Glu Lys Asn
216  Val Ala Thr Ser Glu Gln Asp
223  Lys
224                               Pro Lys Arg Arg Ala Lys Arg Gly Val Ser
234                               Gly Glu Leu Ala Thr Pro Asp Lys Lys Glu
244                               Asn Asp Ala Lys Ser Ser Asp Ser Ser Val
254
```

FIG. 23B

```
                                     Gly Glu Glu Thr Leu Pro Ser Pro Ser Leu
264                                  Asn Met Ala Asn
268                  Glu Ser Gln
271      Thr Glu His Arg Lys Asp
277  Val Asp Glu Tyr
281  Ile Lys Lys Met Leu Ser Glu
288  Ile Gln Leu Asp Arg Arg Lys
295          His Thr Gln Asn Val
300          Asn Leu Asn Ile Lys
305  Leu Ser Ala Ile Lys Thr Lys
312              Tyr Leu Tyr Glu
316  Leu Ser Val Leu Lys Glu Asn
323                      Ser Lys
325  Lys Glu Glu Leu Thr Ser Lys
332              Thr Lys Ala Glu
336  Leu Thr Ala Ala Phe Glu Gln
343  Phe Lys Lys
346                                  Asp Thr Leu Lys Pro
351                  Glu Lys Lys
354  Val Ala Glu Ala Glu Lys Lys
361  Val Glu Glu Ala Lys Lys Lys
368              Ala Lys Asp Gln
372  Lys Glu Glu Asp Arg Arg Asn
```

FIG. 23C

379				Tyr			
380				Pro	Thr	Asn	Thr
384	Tyr	Lys	Thr	Leu	Glu	Leu	Glu
391	Ile	Ala	Glu	Ser	Asp	Val	Lys
398	Val	Lys	Glu	Ala	Glu		
403	Leu	Glu	Leu	Val	Lys	Glu	Glu
410	Ala	Asn	Glu	Ser	Arg	Asn	Glu
417		Glu	Lys	Ile	Lys	Gln	Ala
423	Lys	Glu	Lys	Val	Glu	Ser	Lys
430	Lys	Ala	Glu	Ala	Thr	Arg	
436	Leu	Glu	Lys	Ile	Lys	Thr	Asp
443	Arg	Lys	Lys	Ala	Glu	Glu	Glu
450				Ala	Lys	Arg	Lys
454	Ala	Glu	Glu	Ser	Glu	Lys	Lys
461	Ala	Ala	Glu	Ala	Lys	Gln	Lys
468	Val	Asp	Ala	Glu	Glu	Tyr	Ala
475				Leu	Glu	Ala	Lys
479	Ile	Ala	Glu	Leu	Glu	Tyr	Glu
486	Val	Gln	Arg	Leu	Glu	Lys	Glu

FIG. 23D

493	Leu	Lys	Glu				
496	Ile	Asp	Glu	Ser	Asp	Ser	Glu
503		Asp	Tyr	Leu	Lys	Glu	Gly
509	Leu	Arg	Ala				
512			Pro	Leu	Gln	Ser	Lys
517	Leu	Asp	Thr	Lys	Lys	Ala	Lys
524	Leu	Ser	Lys				
527	Leu	Glu	Glu	Leu	Ser	Asp	Lys
534	Ile	Asp	Glu	Leu	Asp	Ala	Glu
541	Ile	Ala	Lys	Leu	Glu	Val	Gln
548	Leu	Lys	Asp	Ala	Glu	Gly	Asn
555						Asn	Asn
557	Val	Glu	Ala	Tyr	Phe	Lys	Glu
564			Gly	Leu	Glu	Lys	Thr
569			Thr	Ala	Glu	Lys	Lys
574		Ala	Glu	Leu	Glu	Lys	Ala
580	Glu	Ala	Asp	Leu	Lys	Lys	Ala
587	Val	Asp	Glu				

FIG. 23E

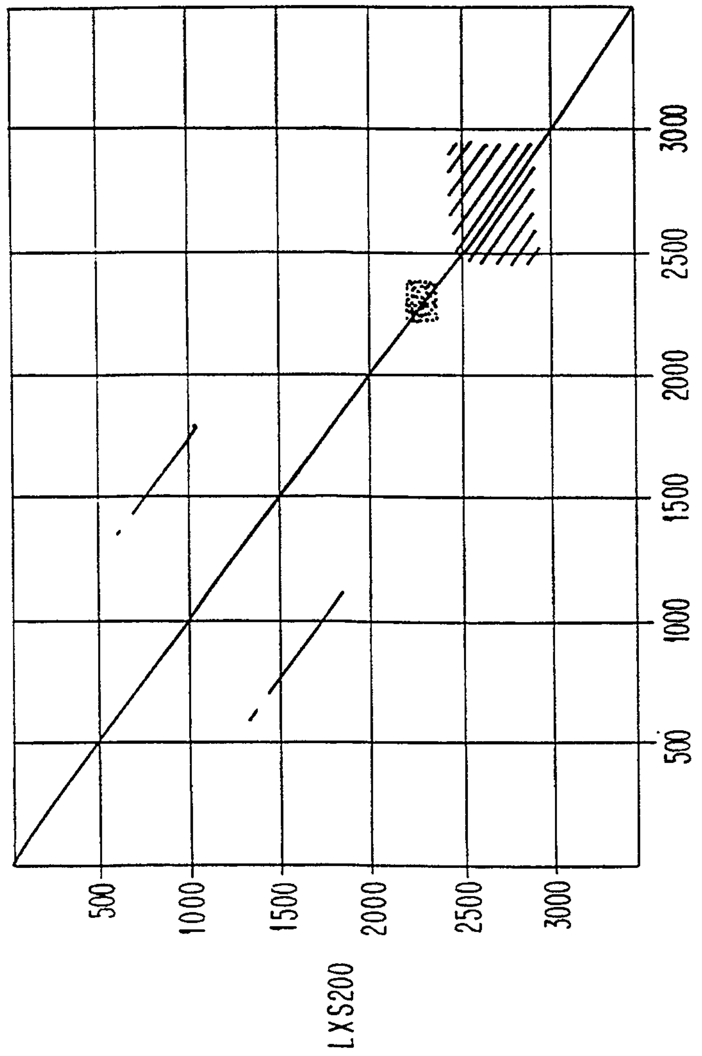

500
1000
1500
2000
2500
3000
LXS200
500
1000
1500
2000
2500
3000
LXS200

FIG. 24

```
  1  CCAAGCTATT AGGTGACACT ATAGAATACT CAAGCTATGC ATCAAGCTTA
 51  TGCTTGTCAA TAATCACAAA TATGTAGATC ATATCTTGTT TAGGACAGTA
101  AAACATCCTA ATTACTTTTT AAATATTCTT CCTGAGTTGA TTGGCTTGAC
151  CTTGTTGAGT CATGCTTATG TGACTTTTGT TTTAGTTTTT CCAGTTTATG
201  CAGTTATTTT GTATCGACGA ATAGCTGAAG AGGAAAAGCT ATTACATGAA
251  GTTATAATCC CAAATGGAAG CATAAAGAGA TAAATACAAA ATTCGATTTA
301  TATACAGTTC ATATTGAAGT AATATAGTAA GGTTAAAGAA AAAATATAGA
351  AGGAAATAAA CATGTTTGCA TCAAAAAGCG AAAGAAAAGT ACATTATTCA
401  ATTCGTAAAT TTAGTATTGG AGTANCTAGT GTAGCTGTTG CCAGTCTTGT
451  TATGGGAAGT GTGGTTCATG CSACCAGARA AACGARGGAA GTACCCAAGC
```

FIG. 25A

```
501   AGCCMCTTCT TCTAATATGG CAAAGACAGA ACATAGGAAA GCYGCTAAAC
551   MAGTCGTCGA TGAATATATA GAAAAAATGT TGAGGGAGAT TCAACTAGAT
601   AGAAGAAAAC ATACCCAAAA TGTCGCCTTA AACATAAAGT TGAGCGCAAT
651   TANAACGAAG TATTTGCGTG AATTAANTGT TNTAGAAGAG AAGTCGAANN
701   ATGAGTTGCC GTCAGAAATA AAAGCGAAGT TAGACGCCGC TTTTGANAAG
751   TTTAAAAAAG ATACATTGAA ACCAGGAGAA AAGGTAGCNG AAGCTAAGAA
801   GAANGTTGAA GAAGCTAAGA AWAAAGCCRA GGATCAAAAA GAAGAAGATC
851   GYCGTAACTA CCCAACCAAT ACTTRCAAAA CGCTTGACCT TGAAATTGCT
901   GAGTYCGATG TGAAAGTTAA AGAAGCGGAG CTTGAACTAG TAAARGAGGA
```

FIG. 25B

```
 951  AGCTMMRGAA YCTCGAGACG AGGAAAAAAT TAAGCAAGCA AAAGCGAAAG
1001  TTGAGAGTAA AAAAGCTGAG GCTACAAGGT TAGAAAACAT CAAGACAGAT
1051  NGTAAAAAAG CAGAAGAAGA AGNTAAACGA AAAGCAGCAG AAGAAGATAA
1101  AGTTAAAGAA AAACCAGCTG AACAACCACA ACCAGCGCCG GNTACTCAAC
1151  CAGAAAAACC AGCTCCAAAA CCAGAGAAGC CAGCTGAACA ACCAAAAGCA
1201  GAAAAAACAG ATGATCAACA AGCTGAAGAA GACTATGCTC GTAGATCAGA
1251  AGAAGAATAT AATCGCTTGA NTCAACAGCA ACCGCCAAAA ACTGAAAAAC
1301  CAGCACAACC ATNTACTCCA AAAACA
```

FIG. 25C

```
              .         .         .         .         .
875   AAAAAGCTAAACTATCAAAACTTGAAGAGTTAAGTGATAAGATTGATGAG 924
      |||| |||  ||  | |||  |||  ||||:   ||  ||  || | ||
877   AAAACGCTTGACCTTGAAA..TTGCTGAGTYCGATGTGAAAGTTAAAGAA 924

              .         .         .         .         .
925   TTAGACGCTGAAATTGCAAAACTTGAAGATCAACTTAAAGCTGCTGAAGA 974
         ||   |||| | | |||:   |||| |:::         |  || ||
925   GCGGAGCTTGAACTAGTAAARGAGGAAGCTMMRGAAYCTCGAGACGAGGA 974

              .         .         .         .         .
975   AAACAATAATGTAGAAGACTACTTTAAAGAAGGTTTAGAGAAAACTATTG 1024
      ||| | |||   || | |       |  ||| ||| ||||
975   AAAAATTAAGCAAGCAAA.......AGCGAAAGTTGAGAG.......... 1007

              .         .         .         .         .
1025  CTGCTAAAAAAGCTGA.........ATTAGAAAAAACTGAAGCTGACCTT 1065
          ||||||||||||          |||||||| |   |  | || : |
1008  ....TAAAAAAGCTGAGGCTACAAGGTTAGAAAACATCAAGACAGATNGT 1053
```

FIG. 26A

```
              .         .         .         .         .
1066 AAGAAAGCAGTTAATGAGCCAGAAAAACCAGCTCCAGCTCCAGAAACTCC 1115
     || |||||||   | ||   ||: |||| |    ||||   ||||  |
1054 AAAAAAGCAG...AAGAAGAAGNTAAACGAAAAGCAGCAGAAGAAGATAA 1100

              .         .         .         .         .
1116 AGCCCCAGAAGCACCAGCTGAACAACCAAAACCAGCGCCGGCTCCTCAAC 1165
     ||    ||||  |||||||||||||||| ||||||||||||:| ||||||
1101 AGTTAAAGAAAAACCAGCTGAACAACCACAACCAGCGCCGGNTACTCAAC 1150

              .         .         .         .         .
1166 CAG...CTCCCGCACCAAAACCAGAGAAGCCAGCTGAACAACCAAAACCA 1212
     |||     || || ||||||||||||||||||||||||||||||||| ||
1151 CAGAAAAACCAGCTCCAAAACCAGAGAAGCCAGCTGAACAACCAAAAGCA 1200

              .         .         .         .         .
1213 GAAAAAACAGATGATCAACAAGCTGAAGAAGACTATGCTCGTAGATCAGA 1262
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1201 GAAAAAACAGATGATCAACAAGCTGAAGAAGACTATGCTCGTAGATCAGA 1250

              .         .         .         .         .
1263 AGAAGAATATAATCGCTTGACTCAACAGCAACCGCCAAAAGCTGAAAAAC 1312
     ||||||||||||||||||||:|||||||||||||||||||| |||||||||
1251 AGAAGAATATAATCGCTTGANTCAACAGCAACCGCCAAAAACTGAAAAAC 1300

              .         .
1313 CAGCTC......CTGCACCAAAAACA 1332
     |||| |      :| | |||||||||
1301 CAGCACAACCATNTACTCCAAAAACA 1326
```

FIG. 26B

```
              .         .         .         .         .
  1 AAGCTTATGCTTGTCAATAATCACAAATATGTAGATCATATCTTGTTTAG 50
    ||||||||||||||||||||||||||||||||||||||||||||||||||
 44 AAGCTTATGCTTGTCAATAATCACAAATATGTAGATCATATCTTGTTTAG 93

              .         .         .         .         .
 51 GACAGTAAAACATCCTAATTACTTTTTAAATATTTTACCTGAGTTGATTG 100
    |||||||||||||||||||||||||||||||||| | |||||||||||||
 94 GACAGTAAAACATCCTAATTACTTTTTAAATATTCTTCCTGAGTTGATTG 143

              .         .         .         .         .
101 GCTTGACCTTGTTGAGTCATGCCTATATGACTTTTGTTTTAGTTTTTCCA 150
    |||||||||||||||||||||| ||| |||||||||||||||||||||||
144 GCTTGACCTTGTTGAGTCATGCTTATGTGACTTTTGTTTTAGTTTTTCCA 193

              .         .         .         .         .
151 GTTTATGCAGTTATTTTGTATCGACGAATAGCTGAAGAGGAAAAGTTATT 200
    ||||||||||||||||||||||||||||||||||||||||||||| ||||
194 GTTTATGCAGTTATTTTGTATCGACGAATAGCTGAAGAGGAAAAGCTATT 243
```

FIG. 27A

```
             .         .         .         .         .
201 ACATGAAGTTATAATCCCAAATGGAAGCATAAAGAGATAAATACAAAATT 250
    ||||||||||||||||||||||||||||||||||||||||||||||||||
244 ACATGAAGTTATAATCCCAAATGGAAGCATAAAGAGATAAATACAAAATT 293

             .         .         .         .         .
251 CGATTTATATACAGTTCATATTGAAGTGATATAGTAAGGTTAAAGAAAAA 300
    |||||||||||||||||||||||||||| |||||||||||||||||||||
294 CGATTTATATACAGTTCATATTGAAGTAATATAGTAAGGTTAAAGAAAAA 343

             .         .         .         .         .
301 ATATAGAAGGAAATAAACATGTTTGCATCAAAAAGCGAAAGAAAAGTACA 350
    ||||||||||||||||||||||||||||||||||||||||||||||||||
344 ATATAGAAGGAAATAAACATGTTTGCATCAAAAAGCGAAAGAAAAGTACA 393

             .         .         .         .         .
351 TTATTCAATTCGTAAATTTAGTATTGGAGTAGCTAGTGTAGCTGTTGCCA 400
    |||||||||||||||||||||||||||||||:||||||||||||||||||
394 TTATTCAATTCGTAAATTTAGTATTGGAGTANCTAGTGTAGCTGTTGCCA 443

             .         .         .         .         .
401 GCTTGTTCTTAGGAGGAGTAGTCCATGCAGAAGGGGTTAGAAGTGGAAT 450
    |  |  |  | ||| | || || |||||   |   |  | | | :||||
444 GTCTTGTTATGGAAGTGTGGTTCATGC..SACCAGARAAACGARGGAAG 491

             .         .         .         .         .
451 AACCTCA....CGGTTACATCTAGTGGGCAAGATATATCGAAGAAGTATG 496
     |||  |    |   | | |||| |  |  | | | |    | | | | |
492 TACCCAAGCAGCCMCTTCTTCTAATATGGCAAAGACAGAACATAGGAAAG 541
```

FIG. 27B

```
             .         .         .         .         .
497 CTGATGAA......GTCGAGTCGCATCTAGAAAGTATATTGAAGGATGTC 540
    |:| | ||      |||||     || ||||||  || |||| |||  |
542 CYGCTAAACMAGTCGTCGATGAATATATAGAAAAAATGTTGAGGGAGATT 591

             .         .         .         .         .
541 AAAAAAAATTTGAAAAAAGTTCAACATACCCAAAATGTCGGCTTAATTAC 590
     ||  | ||   | ||      |||||||||||||||||| |||||  |
592 CAACTAGATAGAAGAA......AACATACCCAAAATGTCGCCTTAAACAT 635

             .         .         .         .         .
591 AAAGTTGAGCGAAATTAAAAAGAAGTATTTGTATGACTTAAAAGTTA... 637
    ||||||||||| |||||:|| ||||||||||  ||| ||||: |||:
636 AAAGTTGAGCGCAATTANAACGAAGTATTTGCGTGAATTAANTGTTNTAG 685

             .         .         .         .         .
638 ATGTTTTATCGGAAGCTGAGTTGACGTCAAAAACAAAAGAAACAAAAGAA 687
    | |     ||| |:: ||||||| ||||         ||||| |||||
686 AAGAGAAGTCGAANNATGAGTTGCCGTC.........AGAAATAAAAGCG 726

             .         .         .         .         .
688 AAGTTAACCGCAACTTTTGAGCAGTTTAAAAAAGATACATTACCAACAGA 737
    ||||||  |||  |||||||: |||||||||||||||||||        |
727 AAGTTAGACGCCGCTTTTGANAAGTTTAAAAAAGATACATT......GAA 770

             .         .         .         .         .
738 ACCAGAAAAAAAGGTAGCAGAAGCTCAGAAGAAGGTTGAAGAAGCTAAGA 787
    ||||| | ||||||||||:|||||| |||||||:||||||||||||||||
771 ACCAGGAGAAAAGGTAGCNGAAGCTAAGAAGAANGTTGAAGAAGCTAAGA 820
```

FIG.27C

```
              .         .         .         .         .
 788 AAAAAGCCGAGGATCAAAAAGAAAAAGATCGCCGTAACTACCCAACCATT 837
     |:||||||:|||||||||||||| |||||||:|||||||||||||||| |
 821 AWAAAGCCRAGGATCAAAAAGAAGAAGATCGYCGTAACTACCCAACCAAT 870

              .         .         .         .         .
 838 ACTTACAAAACGCTTGAACTTGAAATTGCTGAGTCCGATGTGGAAGTTAA 887
     ||||:|||||||||||| |||||||||||||||||:||||||| |||||||
 871 ACTTRCAAAACGCTTGACCTTGAAATTGCTGAGTYCGATGTGAAAGTTAA 920

              .         .         .         .         .
 888 AAAAGCGGAGCTTGAACTAGTAAAAGTGAAAGCTAAGGAATCTCAAGACG 937
     | ||||||||||||||||||||||:| | |||||:::|||:||| |||||
 921 AGAAGCGGAGCTTGAACTAGTAAARGAGGAAGCTMMRGAAYCTCGAGACG 970

              .         .         .         .         .
 938 AGGAAAAAATTAAGCAAGCAGAAGCGGAAGTTGAGAGTAAACAAGCTGAG 987
     |||||||||||||||||||| ||||| |||||||||||||| ||||||||
 971 AGGAAAAAATTAAGCAAGCAAAAGCGAAAGTTGAGAGTAAAAAAGCTGAG 1020

              .         .         .         .         .
 988 GCTACAAGGTTAAAAAAAATCAAGACAGATCGT............GAAGA 1025
     |||||||||||| |||| ||||||||||||:||            |||||
1021 GCTACAAGGTTAGAAAACATCAAGACAGATNGTAAAAAAGCAGAAGAAGA 1070

              .
1026 AGCTAAACGAAAAGCAG 1042
     ||:||||||||||||||
1071 AGNTAAACGAAAAGCAG 1087
```

FIG. 27D

```
                .         .         .         .         .
306 SQTEHRKD....VDEYIKKMLSEIQLDRRKHTQNVNLNIKLSAIKTKYLY 351
    ..|||||.    |||||.|||.|||||||||||||.|||||||| ||||
  2 AKTEHRKAAKXVVDEYIEKMLREIQLDRRKHTQNVALNIKLSAIXTKYLR 51

                .         .         .         .         .
352 ELSVLKENSKKEELTSKTKAELTAAFEQFKKDTLKPEKKVAEAEKKVEEA 401
    || | .|.|   ||.|..||.|.||| .||||||||:.|||||.| ||||
 52 ELXVXEEKS.XXELPSEIKAKLDAAFXKFKKDTLKPGEKVAEAKKXVEEA 100

                .         .         .         .         .
402 KKKAKDQKEEDRRNYPTNTYKTLELEIAESDVKVKEAELELVKEEANESR 451
    | || |||||||||||||| |||:||||| |||||||||||||||| | |
101 KXKAXDQKEEDRRNYPTNTXKTLDLEIAEXDVKVKEAELELVKEEAXEXR 150
```

FIG. 28A

```
             .         .         .         .         .
452 NEEKIKQAKEKVESKKAEATRLEKIKTDRKKAEEEAKRKAEESEKKAAEA 501
    :||||||||.|||||||||||||.|||| |||||| ||||.|.:|
151 DEEKIKQAKAKVESKKAEATRLENIKTDXKKAEEEXKRKAAEEDK..... 195
                             .
                             .
                             .
             .         .         .         .         .
552 SKLDTKKAKLSKLEELSDKIDELDAEIAKLEVQLKDAEGNNNVEAYFKEG 601
                                   |. |.||.
196 ...............................VKEKPAEQ........... 203
             .         .         .         .         .
602 LEKTTAEKKAELEKAEADLKKAVDEPETPAPAPQPAPAPEKPAEKPAPAP 651
                           .:|.. ..:..||| ||||||.| :..
204 .......................PQPAPXTQPEKPAPKPEKPAEQPKAEK 230
             .         .         .         .
652 EKPAPAPEKPAPAPEKP.APAPEKPAPAPEKPAPTPETPKT 691
    .....|.|. |. .|.. ..  :..:| .||||. | ||||
231 TDDQQAEEDYARRSEEEYNRLXQQQPPKTEKPAQ.PXTPKT 270
```

FIG. 28B

```
             .         .         .         .         .
 91 AKKDAKNAKKAVEDAQKALDDAKAAQKKYDEDQKKTEEKAALEKAASEEM 140
    ||.: :.| | | |     :  .   :..: |.:| .:..||:   |.
  2 AKTEHRKAAKXVVD.....EYIEKMLREIQLDRRKHTQNVALNIKLSAIX 46

             .         .         .         .         .
141 DKAVAAVQQAYLAYQQATDKAAKDAADKMIDEAKKREEEAKTKFNTVRAM 190
    .|           | ..    ....  .: .| | : :.|  ||   :
 47 TK...........YLRELXVXEEKSXXELPSEIKAKLDAAFXKF...KKD 82

             .         .         .         .         .
191 VVPEPEQLAETKKKSEEAKQKAPELTKKLEEAKAKLEEAEKKATEAKQKV 240
    .:...|.:||.||  |||| || :         .| |:  . :|:. ..:
 83 TLKPGEKVAEAKKXVEEAKXKAXD.........QKEEDRRNYPTNTXKTL 123
```

FIG.29A

```
             .         .         .         .         .
241 DAEEVAPQAKIAELENQVHRLEQELKEIDESESEDYAKEGFRAPLQSKLD 290
    | | .. :.|: | | ::  :.:|  |  :.|. . ||        .|::
124 DLEIAEXDVKVKEAELEL..VKEEAXEXRDEEKIKQAK........AKVE 163
             .         .         .         .         .
291 AKKAKLSKLEELSDKIDELDAEIAKLEDQLKAAEENNNVEDYFKEGLEKT 340
    .|||. .:||::
164 SKKAEATRLENI...................................... 175
             .         .         .         .         .
341 IAAKKAELEKTEADLKKAVNEPEKPAPAPETPAPEAPAEQPKPAPAPQPA 390
        |.: .|.|.: |: ..|.:|        ..| |||||.||| .||.
176 ....KTDXKKAEEEXKRKAAEEDK........VKEKPAEQPQPAPXTQPE 213
             .         .         .         .         .
391 .PAPKPEKPAEQPKPEKTDDQQAEEDYARRSEEEYNRLTQQQPPKAEKPA 439
     |||||||||||||:||||||||||||||||||||||| ||||||.||||
214 KPAPKPEKPAEQPKAEKTDDQQAEEDYARRSEEEYNRLXQQQPPKTEKPA 263

440 PA..PKT 444
    .:  |||
264 QPXTPKT 270
```

FIG. 29B