(54) Title: PNEUMOLYSIN MUTANTS AND PNEUMOCOCCAL VACCINES MADE THEREFROM

(57) Abstract

Mutants of pneumolysin that are non-toxic by reason of amino acid substitutions have been constructed. These mutants elicit an immune response in animals that is reactive to wild-type pneumolysin. The invention also encompasses vaccines for humans based on these mutants, including vaccines comprising conjugates with pneumococcal capsular polysaccharides.
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PNEUMOLYSIN MUTANTS AND PNEUMOCOCCAL VACCINES
MADE THEREFROM

This invention relates to mutants of the toxin pneumolysin and
pneumococcal vaccines based on these mutants.

BACKGROUND

Streptococcus pneumoniae (pneumococcus) is an important pathogen,
causing invasive diseases such as pneumonia, meningitis and
bacteraemia. Even in regions where effective antibiotic therapy is freely
available, the mortality rate from pneumococcal pneumonia can be as
high as 19% in hospitalized patients and this increases to 30-40% in
patients with bacteraemia. These high mortality rates have been
reported in the U.S.A. where pneumonia, of which S. pneumoniae is
the commonest cause, is the fifth ranking cause of death. Indeed,
pneumonia is the only infectious disease amongst the top ten causes of
death in that country. In the United States mortality rates for
pneumococcal meningitis range from 13-45%. In developing countries,
in excess of 3 million children under the age of 5 years die each year
from pneumonia, and again S. pneumoniae is the commonest
causative agent. S. pneumoniae also causes less serious, but highly
prevalent infections such as otitis media and sinusitis, which have a
significant impact on health-care costs in developed countries. Otitis
media is especially important in young children; sinusitis affects both
children and adults.

In the late 1970's, a vaccine was licensed for the purpose of preventing
serious infections, especially bacterial pneumonia and for protecting
certain groups, such as splenectomized individuals and young children,
who are particularly susceptible to fulminating pneumococcal disease.
The vaccine is composed of purified capsular polysaccharides, which
are the predominant pneumococcal surface antigens. However, each
serotype of S. pneumoniae (of which there are 83) has a structurally
distinct capsular polysaccharide, and immunization with one serotype
confers no protection whatsoever against the vast majority of the others.
The vaccine currently licensed in Australia contains polysaccharides
purified from the 23 most common serotypes, which account for
approximately 90% of pneumococcal infections in this country.
Protection even against those serotypes contained in the vaccine is by no means complete, and there have been several reports of serious, even fatal infections occurring in vaccinated high-risk individuals. The efficacy of the vaccine is poorest in young children, and several studies, including one conducted in Adelaide, have shown that the existing formulation has little or no demonstrable clinical benefit in this group. This apparent failure of the vaccine appears to be related to the poor immunogenicity of certain pneumococcal polysaccharides in children under 5 years of age. We have shown that the antibody response is particularly poor to the five serotypes which most commonly cause disease in children (types 6, 14, 18, 19 and 23). Indeed, the antibody response to these pneumococcal polysaccharides only approaches adult levels in children over 8 years of age at the time of vaccination.

In view of this, a vaccine, including antigens other than the capsular polysaccharides seems to be required to protect young children from pneumococcal infection. One such antigen could be pneumolysin, a protein toxin produced by all virulent *S. pneumoniae* isolates. Immunization of mice with this protein has been found to confer a degree of protection from pneumococcal infection.

However there is a difficulty in that pneumolysin is toxic to humans. Thus pneumolysin included in a vaccine must therefore be substantially non-toxic. However, the rendering of a pneumolysin non-toxic by most currently employed methods would be likely to alter the basic configuration of the protein so as to be immunogenically distinct from the native or wild-type pneumolysin. An immune response elicited by an altered protein that is immunogenically distinct from the native pneumolysin will have a decreased protective capacity or no protective capacity. Thus the difficulty is to produce an altered pneumolysin that is non-toxic and at the same time sufficiently immunogenically similar to the toxic form to elicit a protective immune response.

An altered pneumolysin with the above characteristics can then be used in a number of ways in a vaccine. Thus the altered pneumolysin may be used by itself to immunise, or alternatively the altered pneumolysin may be conjugated to pneumococcal polysaccharide, or
alternatively may be included in a vaccine wherein pneumococcal polysaccharides may be conjugated to another protein and the altered pneumolysin is present in a non-conjugated form only. Alternatively, pneumococcal polysaccharide and pneumolysin may both be used in an unconjugated form.

DESCRIPTION OF INVENTION
In a broad form therefore the invention may be said to reside in an altered pneumolysin being substantially non-toxic and being capable of eliciting an immune response in an animal being reactive to wild-type pneumolysin.

Preferably the altered pneumolysin has reduced complement binding activity as compared to wild-type pneumolysin. Reduction in the complement binding activity results in less inflammation at the site of administering the vaccine.

Preferably the altered pneumolysin has reduced Fc binding activity as compared to wild-type pneumolysin. Reduction in the Fc binding activity results in less inflammation at the site of administering the vaccine.

Preferably the altered pneumolysin is altered by reason of one or more amino acid substitutions relative to wild-type pneumolysin.

The pneumolysin may be altered in that the amino acid present at any one or more than one of residue sites 367, 384, 385, 428, 433 or 435 of wild-type pneumolysin are replaced, removed or blocked.

In a further form the invention could be said to reside in a vaccine including an altered pneumolysin, said altered pneumolysin being non-toxic and being capable of eliciting an immune response in an animal being reactive to wild-type pneumolysin.

Preferably the vaccine comprises capsular polysaccharide material conjugated with the altered pneumolysin.
The capsular material may be derived from any one or more of the *Streptococcus pneumoniae* serotypes 6A, 6B, 14, 18C, 19A, 19F, 23F, 1, 2, 3, 4, 5, 7F, 8, 9N, 9V, 10A, 11A, 12F, 15B, 17F, 20, 22F and 33F.

In this embodiment serotypes which are commonly associated with disease in children, and to which children generally have a poor immune response, may be specifically targeted (i.e. Danish serotypes 6A, 6B, 14, 18C, 19A, 19F and 23F). Other common serotypes contained in the present 23-valent Merck Sharp and Dohme vaccine (Pneumovax 23) however, could also be used to synthesize conjugates (i.e. types 1, 2, 3, 4, 5, 7F, 8, 9N, 9V, 10A, 11A, 12F, 15B, 17F, 20, 22F and 33F) or indeed any other serotype. Conjugation of any pneumococcal polysaccharides to the protein carrier ensures good T-cell dependent immunogenicity in children, such that protective levels of anti-polysaccharide antibody are produced.

The combination of the altered pneumolysin together with the capsular material will ensure an extra degree of protection, particularly against serotypes of *S. pneumoniae* whose polysaccharides are not incorporated in the existing vaccine formulations.

The vaccine is preferably administered by sub-cutaneous injection, with or without an approved adjuvant, such as alumina gel.

In another form the invention could be said to reside in a recombinant clone including a replicon and a DNA sequence encoding an altered pneumolysin, said altered pneumolysin being non-toxic and being capable of eliciting an immune response in an animal being reactive to wild-type pneumolysin.

In yet another form the invention could be said to reside in a method of producing an altered pneumolysin including the steps of purifying said altered pneumolysin from an expression system including a recombinant clone with DNA encoding an altered pneumolysin said pneumolysin being substantially non-toxic and being capable of eliciting an immune response in an animal reactive to wild-type pneumolysin.
Preferably the expression system is a culture of a host cell including a recombinant clone with DNA encoding the altered pneumolysin.

In another form the invention could be said to reside in a method of producing a vaccine including the step of amplifying a recombinant clone encoding an altered pneumolysin, inducing transcription and translation of said cloned material, the purification of altered pneumolysin, and the step of conjugating the altered pneumolysin with a capsular polysaccharide, the altered pneumolysin having substantially reduced toxic activity as compared with wild-type pneumolysin.

For a better understanding of the invention specific embodiments of the invention will now be described with reference to diagrams wherein:

**FIG. 1** is the DNA sequence of the gene encoding wild-type pneumolysin,

**FIG. 2** is the DNA sequence of an altered gene encoding wild type pneumolysin used for cloning the pneumolysin gene into an expression vector,

**FIG. 3** is the amino acid sequence of the wild-type pneumolysin as derived from the DNA sequence of the gene encoding the wild type pneumolysin, and

**FIG. 4** shows the amino acid sequence of pneumolysin showing amino acid substitutions introduced by site directed mutagenesis.

Recombinant DNA techniques have been used to construct non-toxic pneumolysin derivatives suitable for administration to humans. To achieve this, the *S. pneumoniae* gene encoding pneumolysin was cloned into *Escherichia coli* and its complete DNA sequence determined. The DNA sequence is shown in Figure 1 and the derived amino acid sequence is shown in Figure 3.
Three regions of the pneumolysin gene were subjected to oligonucleotide-directed mutagenesis. The first region encodes amino acids 427 - 437 in the protein sequence, and is indicated by an underline in Figure 3. This 11 amino acid sequence shows absolute homology with similar regions in other related thiol activated toxins thus is thought to be responsible for the haemolytic activity and hence toxic activity of the toxin. The other two regions encode amino acids 257 - 297 and amino acids 368 - 397 and are also indicated by an underline in Figure 3. These two regions of the toxin have substantial amino acid sequence homology with human C-reactive protein (CRP), and by inference therefore, are thought to be responsible for the ability of pneumolysin to bind the Fc region of immunoglobulins and to activate complement. Fifteen separate mutations in the pneumolysin gene, resulting in single amino acid substitutions, were constructed, as shown in Figure 4. In an effort to maintain the structure of the altered pneumolysin, conservative substitutions were made, so that amino acids are substituted with amino acids of a similar nature.

For the region involved in haemolytic activity, Cys 428 -> Gly, Cys 428 -> Ser, Trp 433 -> Phe, Glu 434 -> Asp and Trp 435 -> Phe each reduced haemolytic activity by 97%, 90%, 99%, 75% and 90% respectively. The other mutations in that region (Cys 428 -> Ala, Glu 434 -> Gln and Trp 436 -> Phe) did not affect haemolytic activity. Mutating a separate region of the toxin thought to be responsible for binding to target cell membranes also affects haemolytic activity of the protein. This substitution, His 367 -> Arg, completely inhibits haemolytic activity. This is a quite unpredictable finding in that His 367 -> Arg therefore shows a greater inhibition of this property than the substitutions made within the 11 amino acid region thought to be responsible for haemolytic activity.

Mutations in the CRP-like domains were tested for ability to activate complement. For Trp 379 -> Phe, Tyr 384 -> Phe, Asp 385 -> Asn, and Trp 397 -> Phe, complement activation was reduced by 20%, 70%, 100% and 15%, respectively. The other mutations in the CRP-like domains shown in Figure 4 do not reduce complement activation.
Importantly, the above mutations which affect either haemolytic activity or complement activation do not impair the immunogenicity of the proteins, compared with native or wild-type pneumolysin.

Thus although His$_{367}$ -> Arg is the preferred mutation to reduce the haemolytic activity, a combination of two or more mutants effecting reduced haemolytic activity can also achieve a very high level of reduction in haemolytic activity. Similarly Asp$_{385}$ -> Asn is the preferred mutation to achieve reduced complement activation, however a combination of two or more other mutants that reduce the activity to a lesser degree can also be used.

In a preferred embodiment the pneumolysin derivative for use in the vaccine would contain a combination of certain of the above mutations such that the protein is unable to activate complement in addition to having zero haemolytic activity. Examples of such combination are:-

1) His$_{367}$ -> Arg + Asp$_{385}$ -> Asn,
2) His$_{367}$ -> Arg + Asp$_{385}$ -> Asn + either Cys$_{428}$ -> Gly or Trp$_{433}$ -> Phe
3) Asp$_{385}$ -> Asn + Cys$_{428}$ -> Gly + Trp$_{433}$ -> Phe

These then are some preferred combinations, however it is to be understood that other combinations of mutations can be used to make up the altered pneumolysin for use in a vaccine. Further the altered pneumolysin may comprise any one of the individual mutations with sufficiently reduced activity.

High level expression of the altered pneumolysin from DNA encoding the altered pneumolysin can be achieved by using any one of a number of conventional techniques including the expression in a prokaryotic host with the DNA cloned appropriately within any one of the many expression vectors currently available, or cloned appropriately within the host chromosome; expression in a eukaryotic host with the DNA cloned appropriately either within an expression vector or cloned within the host chromosome; or within an in vitro expression system such as may comprise purified components necessary for expression of altered pneumolysin.
To achieve high level expression of the mutated pneumolysin gene, it has been cloned into the vector pKK233-2 for expression within *Escherichia coli* or other like prokaryote. This vector included ampicillin and tetracycline resistance genes, the trc promoter (which can be regulated by IPTG [isopropyl-β-D-thiogalactopyranoside]), and a lac Z ribosome binding site adjacent to an ATG initiation codon incorporating an Ncol restriction site. Immediately downstream from the initiation codon there are restriction sites for *PstI* and *HindIII*, followed by a strong T1 T2 transcription terminator. Prior to insertion into pKK233-2, a Ncol restriction site was constructed at the 5' end of the pneumolysin coding sequence (at the initiation codon) by oligonucleotide-directed mutagenesis, as shown in Figure 2. This enabled the proximal end of the altered pneumolysin gene to be cloned into the Ncol site of pKK233-2; a *HindIII* site approximately 80 bases downstream from the pneumolysin termination codon was used to splice the distal end of the altered gene into the compatible site in pKK233-2. The mutant pneumolysin derivative could however, be cloned into any one of a number of high expression vector systems.

The mutant pneumolysin is prepared as follows: *E. coli* cells harbouring the above recombinant plasmid are first grown in 9 litre cultures in Luria Bertani (or any other appropriate) medium, supplemented with the appropriate antibiotic, at 37°C, with aeration. When the culture reaches the late logarithmic phase of growth, IPTG is added to a final concentration of 20μM (to induce expression of the altered pneumolysin gene) and incubation is continued for a further 2 to 3 hours.

Cells are then harvested by centrifugation or ultrafiltration and lysed by treatment with EDTA and lysozyme, followed by sonication, or by disruption in a French pressure cell. Cell debris is removed by centrifugation and the extract is then dialysed extensively against 10mM sodium phosphate (pH7.0). The material is then loaded onto a column of DEAE-cellulose and eluted with a linear gradient of 10-250mM sodium phosphate (pH7.0). Fractions containing peak levels of the pneumolysin derivative are pooled, concentrated by ultrafiltration and loaded onto a column of Sephacryl S-200. This column is developed in 50mM sodium phosphate (pH7.0) and again fractions with high levels of pneumolysin derivative are pooled, concentrated by
ultrafiltration and stored in 50% glycerol at -15°C. The final product is greater than 95% pure, as judged by SDS-polyacrylamide gel electrophoresis. Hydrophobic interaction chromatography on Phenyl-Sepharose is an alternative purification which could also be used.

However it is to be understood that this is only one method of purification of the altered pneumolysin, and other, alternative methods (including High Pressure Liquid Chromatography) may be employed.

This purified altered pneumolysin can then be administered as a vaccine at appropriate levels, either by itself or in combination with other antigens. In one form the pneumolysin may be conjugated with polysaccharide derived from any one or more of the variety of pneumococcal strains described above.

The mutant pneumolysin can be conjugated to the various serotypes of polysaccharide by a range of methods. The first involves preparation of an activated polysaccharide by treating pure polysaccharide (available commercially) with cyanogen-bromide and adipic acid dihydrazide (ADH). The ADH-polysaccharide is then combined with the mutant pneumolysin in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide - HCl. Conjugated material is separated from the reactants by chromatography through Sepharose CL-4B.

Alternatively, the polysaccharide-mutant pneumolysin conjugates can be prepared using bifunctional reagents such as N-succinimidyl-6(4'-azido-2'-nitropheny)hexanoate (SANPAH). Pure polysaccharide dissolved in phosphate buffered saline, is reacted with SANPAH in the presence of a strong white light source. Unreacted SANPAH is then separated from activated polysaccharide by chromatography on Sephadex G-50. Activated polysaccharide is then conjugated to the mutant pneumolysin in 0.2M borate buffer (pH8.5). Any excess reactive groups are then blocked with lysine, and the polysaccharide-protein conjugate is separated from the other reactants by chromatography on Sepharose CL-4B. Conjugates could also be prepared by reductive amination with cyanoborohydride.
Alternatively another protein, such as inactivated tetanus toxin, can be conjugated with the desired polysaccharides and altered pneumolysin can be added to the vaccine in an unconjugated form.

This then describes the best method of performing the invention however it is to be understood that the invention is not limited thereto.
1. An altered pneumolysin being substantially non-toxic and being capable of eliciting an immune response in an animal being reactive to wild-type pneumolysin.

2. An altered pneumolysin as in claim one having reduced complement binding activity as compared to wild-type pneumolysin.

3. An altered pneumolysin as in any one of claims 1 or 2 having reduced Fc binding activity as compared to wild-type pneumolysin.

4. An altered pneumolysin as in any one of claims 1, 2, or 3 wherein said altered pneumolysin is altered by reason of one or more amino acid substitutions within wild type pneumolysin.

5. An altered pneumolysin having the following amino acid sequence:-

\[
\begin{align*}
\text{Met} & \text{ Ala Asn Lys Ala Val Asn Asp Phe Ile Leu Ala Met} \\
& \text{Asn Tyr Asp Lys Lys Leu Leu Thr His Gln Gly Glu} \\
& \text{Ser Ile Glu Asn Arg Phe Ile Lys Glu Gly Asn Gln Leu} \\
& \text{Pro Asp Glu Phe Val Val Ile Glu Arg Lys Lys Arg Ser} \\
& \text{Leu Ser Thr Asn Thr Ser Asp Ile Ser Val Thr Ala Thr} \\
& \text{Asn Asp Ser Arg Leu Tyr Pro Gly Ala Leu Leu Val Val} \\
& \text{Asp Glu Thr Leu Leu Glu Asn Asn Pro Thr Leu Leu Ala} \\
& \text{Val Asp Arg Ala Pro Met Thr Tyr Ser Ile Asp Leu Pro} \\
& \text{Gly Leu Ala Ser Ser Asp Ser Phe Leu Gln Val Glu Asp} \\
& \text{Pro Ser Asn Ser Ser Val Arg Gly Ala Val Asn Asp Leu} \\
& \text{Leu Ala Lys Trp His Gln Asp Tyr Gly Gln Val Asn Asn} \\
& \text{Val Pro Ala Arg Met Gin Tyr Glu Lys Ile Thr Ala His} \\
& \text{Ser Met Glu Gin Leu Lys Val Lys Phe Gly Ser Asp Phe} \\
& \text{Glu Lys Thr Gly Asn Ser Leu Asp Ile Asp Phe Asn Ser} \\
& \text{Val His Ser Gly Glu Lys Gin Ile Gln Ile Val Asn Phe}
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wherein R₁ is His or Arg, R₂ is Trp or Phe, R₃ is Tyr or Phe, R₄ is Asp or Asn, R₅ is Trp or Phe, R₆ is Cys, Gly, or Ser, R₇ is Trp or Phe, R₈ is Glu, or Asp, R₉ is Trp or Phe, and wherein at least one of the residues R₁, R₆, R₇, R₈, or R₉ is other than wild-type.
6. An altered pneumolysin as in claim 5 wherein
wherein R₁ is Arg, R₂ is Trp, R₃ is Tyr, R₄ is Asn, R₅ is Trp, R₆ is Cys, R₇ is Trp, R₈ is Glu, and R₉ is Trp.

5 7. A vaccine including an altered pneumolysin, said altered pneumolysin being non-toxic and being capable of eliciting an immune response in an animal being reactive to wild-type pneumolysin.

8. A vaccine as in claim 7 wherein the altered pneumolysin is as claimed in any one of claims 2 to 6.

9. A vaccine comprising capsular polysaccharide material conjugated with a protein carrier and non-conjugated protein material, the capsular polysaccharide material being derived from any one or more than one of the Streptococcus pneumoniae serotypes, and the non-conjugated protein material being an altered pneumolysin, said altered pneumolysin being non-toxic and being capable of eliciting an immune response in an animal reactive to wild type pneumolysin.

10. A vaccine as in claim 9 wherein the capsular material is derived from any one or more of the Streptococcus pneumoniae serotypes 6A, 6B, 14, 18C, 19A, 19F, 23F, 1, 2, 3, 4, 5, 7F, 8, 9N, 9V, 10A, 11A, 12F, 15B, 17F, 20, 22F and 33F.

11. A vaccine as in either claim 9 or 10 wherein the altered pneumolysin is as claimed in as in any one of claims 2 to 6.

12. A vaccine comprising capsular polysaccharide material conjugated with a protein carrier, the capsular polysaccharide material being derived from any one or more than one of the Streptococcus pneumoniae serotypes, and the protein carrier being an altered pneumolysin, said altered pneumolysin being non-toxic and being capable of eliciting an immune response in an animal reactive to wild type pneumolysin.

13. A vaccine as in claim 12 wherein the capsular material is derived from any one or more of the Streptococcus pneumoniae serotypes 6A,
6B, 14, 18C, 19A, 19F, 23F, 1, 2, 3, 4, 5, 7F, 8, 9N, 9V, 10A, 11A, 12F, 15B, 17F, 20, 22F and 33F.

14. A vaccine as in either claim 12 or 13 wherein the altered pneumolysin is as claimed in any one of claims 2 to 6.

15. A recombinant plasmid including a DNA sequence encoding an altered pneumolysin as claimed in any one of claims 1 to 6.

16. A hybrid host cell including a recombinant plasmid as claimed in claim 9 said recombinant plasmid including an inducible expression control operable for expression of said altered pneumolysin encoding DNA within a host cell.

17. A method of producing an altered pneumolysin including the steps of purifying said altered pneumolysin from an expression system including a recombinant plasmid with DNA encoding an altered pneumolysin said pneumolysin being substantially non-toxic and being capable of eliciting an immune response in an animal reactive to wild type pneumolysin.

18. A method of producing an altered pneumolysin including the steps of purifying said altered pneumolysin from a culture of a host cell including a recombinant clone with DNA encoding an altered pneumolysin said pneumolysin being substantially non-toxic and being capable of eliciting an immune response in an animal said immune response being reactive to wild type pneumolysin.

19. A method of producing a vaccine including the step of amplifying a recombinant clone encoding an altered pneumolysin, inducing transcription and translation of said cloned material, the purification of altered pneumolysin, and the step of conjugating the altered pneumolysin with a capsular polysaccharide, the altered pneumolysin having substantially reduced toxic activity as compared with wild type pneumolysin.

20. A method of producing a vaccine as in claim 19 wherein said altered pneumolysin is as claimed in any one of claims 2 to 6.
21. An altered pneumolysin as hereinbefore described with reference to the examples.

22. A vaccine including an altered pneumolysin as hereinbefore described with reference to the examples.

23. A method of producing a vaccine as hereinbefore described with reference to the examples.
AGATGGCAAA TAAAGCAGTA AATGACTTTA TACTAGCTAT GAATTACGAT
AAAAGAAAC TCTTGACCCA TCAGGGAGAA AGTATTGAAA ATCGTTTCAT
CAAAGGAGGT AATCAGCTAC CGGATGAGTT TGGTGTATTC GAAAGAAGA
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GACAGTGCC TCTATCCCTG AGCCTTTCTC GTAATGAGTG AGACCTTGGT
AGAGAATAAT CCCACTCTTC TTGCGGTGGA TCGTGTCGCC ATGACTTATA
GTATTTGTAT GCCTGGTTTG GCAAGTAGCG ATAGCTTCTC CCAAGTGGAA
GACCCCCAGCA ATTCAAGTGT TGCGGAGACG GTAACGATT TGTTGGCTAA
GTGGCATCAA GATTATGGTC AGGTCAATAA TGTCACAGCT AGAAATGAGT
ATGAAAAAAAAT AACGGCTCAC AGCATGGAAC AACTCAAGGT CAAGTTTGTG
TCTGACTTTTG AAAAGACAGG GAATTCCTTT GATATTGATT TTAACCTCTG
CCATTCAAGT GAAAAGCAAG TCCAGATGTT TAATTTTAAG CAGATTATT
ATRCAGTCAG CGTAGACGCT GTAAAAATCT CAGGAGATGT GTTTCAAGAT
ACTGTAAACGG TAGAGGATTG AAAACAGAGA GGAATTCTCTG CAGAGCGTCC
TTTTGGCTAT ATTTCCAGTG TGCTTTATGG GCCCAAGTGC TATCTCAAGT
TGGAAAAACG GATGAAGGTT GATGAAATGG AGGCTGCTTT GGAAGGTTTG
ATAAAAAGGAG TCAAGTGGGC TCTCAGACCA GAGTGGAAGC AGATTTTGGGA
CAATACAGAA GTGAAGGCGG TTATTTTAGG GGGCGACCCA AGTTCGCGGTG
CCCGAGTTGT AACAGGCAAG GTGGATATGG TAGAGACCTT GATTCAGAGA
GGCGATCGCT TTACAGCAGA TCATCCAGGC TTGCCGATTG CCTATAAAC
TTTTTTTTTA CGTGACAATT TAGTGCGAC CTTCACACAC AGTACGACT
ATGTTTACGAC TAAGGTTACA GCTTACAGAA ACGGAGATTT ACTGCGGGAT
CATAGGGTG CCTATGTTGC CCAATATTAT ATTACCGGGA ATGAATATAC
CTTGATCAT CAAGGTAAGG AAGTCTTGAC TCTAAGGCT TGGGACAGAA
ATGGGCGAGA TTTGACGGCT CATTTTACCA CATGATATCC TTTAAAGGG
AATGTTTCGTA ATCTCTCTGT CAAATAAGA GAGTTGACCG GGCTTGCCCTG
GGAATGGGTG CGTACGGTTT ATGAAAAAC CGATTTGCCA CTAGTGCGTA
AGCGGACGAT TTCTATTGG GGAACACCTC TCTATCTCA GTAGAGGAT
AAGGTAGAAA ATGAC

FIGURE 1 DNA sequence of pneumolysin gene. ATG start codon underlined
CCATGGCATA TAAAGCAGTA AATGACTTTTA TACTAGCTAT GAATTACGAT
AAAAAGAAAC TCTTGACCCA TCAGGGAGAA AGTATGGAAA ATCGTTTCAT
CAAAAGAGGT AACACGTCTAC CCAGTACATT TGTTGTGATC GAAAGAAAGA
AGCGGAGCTT GTGCACAAAT ACAAGTGATA TTTCTGTAAAC AGCTACCAAC
GACAGCGGCT TCTATCTCTG AGCACTTTCT GTAGTGGAGTC AGACCTTGGT
AGAGAAATAT CCCACTCTTC TTGCGGTTGA TCGTGCTCCG ATGACCTTATA
GTATGGTTTG GCCCTGTTTG GCAAATGAGCG ATAGGTTTCTTC CCAAGTGGGA
GACCCCAGCA ATTCAAGTGT TCGGCGAGGC GTAAACGATT TGGTGGCTAA
GTCGCATCAA GATTATGTCG AGGTCAATAA TGTCGAGCCT AGAATGCGT
ATGAAAAAAT AACGCGCTCAC AGCATGGAAC AACTCAAGGT CAAAGTTTGGT
TCTGACTTTG AAAAGACAGG GAATTCTCCT GATATGGATT TTAACCTCTGT
CCATTCAAGGT GAAAGCAAGA TTCAGATTGT TAAATTAAAG CAGATTATT
ATACAGTCAG CGTAGACGCCT GTTAAAAATC CAGGAGATGT GTTTCACAGAT
ACTGTAACGG TAGAGGATTTC AAAACAGAGA GGAATTTCTA CAGAGCCTCC
TTTGCTCTAT ATTTTGAGTG TGGCTTATGG GCCCAAGCTC TATCTCAAGT
TGGAAACCAC GAGTAAGAGT GATGAAGTAG AGGCTGCTTT TGAACTCTTG
ATAAAAAGGAG TCAAGGTAAG TCCTCAAGCA GAGTGGAAGG AGATTTTGGA
CAATACGAAA GTGAAAGGCGG TTAATTGAAG GGGCGACCCG AGTTCCGGTG
CCGAGTTTGT AACAGGCGAG GTGGATATGG TAGAGGACTT GATTCAAGAA
GCCAGTGCCT TTAGACGAGA TCATTCAGGC TTGCAGATTT CCTATAACAAC
TTCTTTTTTA CGTGACATGT TAGTTGCGAC CTTTCAAAAC AGTACGAGCT
ATGGTTGAGC TAGGGTACA GCTTACAGAA ACGGAGATT TACTGCTGAT
CATAGTGCTT CCTATGTGTC CCATATATAT ATTACTTGGG ATGAATTATC
CTATGATCAG CAAAGGCAAG AAGTCTTGAC TCCTAAGGCT TGGGACAGAA
ATGGCCAGGA TTTGGACGGCT CACCTTATCA CTAAGTATCC TTTAAAAGGG
AAGTGTGGTA ATCTCTCTGT CAAATAATGA GAGTGACC GGCTTGCGTG
GGAATGGTGG CGTAGGCGTTT ATGAAAAAAC CGATTTGCCA CTAAGTGCGTA
AGCGGAGCAT TTCTATTTGG GGAACAATCT TCTATCCTCA GTAGAGGAT
AAGGTAAGAA ATGAC

FIGURE 2 DNA sequence of modified pneumolysin gene. An NcoI restriction site (underlined) has been introduced at the start codon.
Met Ala Asn Lys Ala Val Asn Asp Phe Ile Leu Ala Met
1
Asn Tyr Asp Lys Lys Leu Leu Thr His Gin Gly Glu
21
Ser Ile Glu Asn Arg Phe Ile Lys Glu Gly Asn Gin Leu
31
Pro Asp Glu Phe Val Val Ile Glu Arg Lys Lys Arg Ser
41
Leu Ser Thr Asn Thr Ser Asp Ile Ser Val Thr Ala Thr
61
Asn Asp Ser Arg Leu Tyr Pro Gly Ala Leu Leu Val Val
71
Asp Glu Thr Leu Leu Glu Asn Asn Pro Thr Leu Leu Ala
81
Val Asp Arg Ala Pro Met Thr Tyr Ser Ile Asp Leu Pro
101
Gly Leu Ala Ser Ser Asp Ser Phe Leu Gin Val Glu Asp
111
Pro Ser Asn Ser Ser Val Arg Gly Ala Val Asn Asp Leu
121
Leu Ala Lys Trp His Gin Asp Tyr Gly Gin Val Asn Asn
131
Val Pro Ala Arg Met Gln Tyr Glu Lys Ile Thr Ala His
151
Ser Met Glu Gln Leu Lys Val Lys Phe Gly Ser Asp Phe
161
Glu Lys Thr Gly Asn Ser Leu Asp Ile Asp Phe Asn Ser
171
Val His Ser Gly Glu Lys Gin Ile Gin Ile Val Asn Phe
191
Lys Gln Ile Tyr Tyr Thr Val Ser Val Asp Ala Val Lys
201
Asn Pro Gly Asp Val Phe Gin Asp Thr Val Thr Val Glu
211
Asp Leu Lys Gln Arg Gly Ile Ser Ala Glu Arg Pro Leu
231
Val Tyr Ile Ser Ser Val Ala Tyr Gly Arg Gln Val Tyr
241
Leu Lys Leu Glu Thr Thr Ser Lys Ser Asp Glu Val Glu
251
Ala Ala Phe Glu Ala Leu Ile Lys Gly Val Lys Val Ala
261
Pro Gln Thr Glu Trp Lys Gln Ile Leu Asp Asn Thr Glu
271
Val Lys Ala Val Ile Leu Gly Gly Asp Pro Ser Ser Gly
281
Ala Arg Val Val Thr Gly Lys Val Asp Met Val Glu Asp
291
Leu Ile Gln Glu Gly Ser Arg Phe Thr Ala Asp His Pro
301
Gly Leu Pro Ile Ser Tyr Thr Thr Ser Phe Leu Arg Asp
311
Asn Val Val Ala Thr Phe Gln Asn Ser Thr Asp Tyr Val
321
Glu Thr Lys Val Thr Ala Tyr Arg Asn Gly Asp Leu Leu
331
Leu Asp His Ser Gly Ala Tyr Val Ala Glu Tyr Tyr Ile
341
Thr Trp Asp Glu Leu Ser Tyr Asp His Gln Gly Lys Glu
351
Val Leu Thr Pro Lys Ala Trp Asp Arg Asn Gly Gln Asp
361
Leu Thr Ala His Phe Thr Thr Ser Ile Pro Leu Lys Gly
371
Asn Val Arg Asn Leu Ser Val Lys Ile Arg Glu Cys Thr
381
Gly Leu Ala Trp Glu Trp Trp Arg Thr Val Tyr Glu Lys
391
Thr Asp Leu Pro Leu Val Arg Lys Arg Thr Ile Ser Ile
401
Figure 3
Met Ala Asn Lys Ala Val Asn Asp Phe Ile Leu Ala Met 1

Asn Tyr Asp Lys Lys Leu Leu Thr His Glu Gly Glu 21

Ser Ile Glu Asn Arg Phe Ile Lys Glu Gly Asn Gln Leu 31

Pro Asp Glu Phe Val Val Ile Glu Arg Lys Lys Arg Ser 41

Leu Ser Thr Asn Thr Ser Asp Ile Ser Val Thr Ala Thr 61

Asn Asp Ser Arg Leu Tyr Pro Gly Ala Leu Leu Val Val 71

Asp Glu Thr Leu Leu Glu Asn Asn Pro Thr Leu Leu Ala 81

Val Asp Arg Ala Pro Met Thr Tyr Ser Ile Asp Leu Pro 101

Gly Leu Ala Ser Ser Asp Ser Phe Leu Gln Val Glu Asp 111

Pro Ser Asn Ser Ser Val Arg Gly Ala Val Asn Asp Leu 121

Leu Ala Lys Trp His Gln Asp Tyr Gly Gln Val Asn Asn 131

Val Pro Ala Arg Met Gln Tyr Glu Lys Ile Thr Ala His 151

Ser Met Glu Gln Leu Lys Val Lys Phe Gly Ser Asp Phe 161

Glu Lys Thr Gly Asn Ser Leu Asp Ile Asp Phe Asn Ser 171

Val His Ser Gly Glu Lys Gln Ile Gln Ile Val Asn Phe 191

Lys Gln Ile Tyr Tyr Thr Val Ser Val Asp Ala Val Lys 201

Asn Pro Gly Asp Val Phe Gln Asp Thr Val Thr Val Glu 211

Asp Leu Lys Gln Arg Gly Ile Ser Ala Glu Arg Pro Leu 231
Val Tyr Ile Ser Ser Val Ala Tyr Gly Arg Gin Val Tyr
241
Leu Lys Leu Glu Thr Thr Ser Lys Ser Asp Glu Val Glu
251
\[ \text{Trp} \]
\[ \text{Ala Ala Phe Glu Ala Leu Ile Lys Gly Val Lys Val Ala} \]
261
\[ \text{Phe} \]
Pro Gln Thr Glu Trp Lys Gln Ile Leu Asp Asn Thr Glu
281
Val Lys Ala Val Ile Leu Gly Gly Asp Pro Ser Ser Gly
291
Ala Arg Val Val Thr Gly Lys Val Asp Met Val Glu Asp
301
Leu Ile Gin Glu Gly Ser Arg Phe Thr Ala Asp His Pro
321
\[ \text{Gly Leu Pro Ile Ser Tyr Thr Thr Ser Phe Leu Arg Asp} \]
331
Asn Val Val Ala Thr Phe Gln Asn Ser Thr Asp Tyr Val
341
Glu Thr Lys Val Thr Ala Tyr Arg Asn Gly Asp Leu Leu
351
\[ \text{Arg} \]
Leu Asp His Ser Gly Ala Tyr Val Ala Gin Tyr Tyr Ile
361
\[ \text{Phe} \]
Phe Asn
371
Thr Trp Asp Glu Leu Ser Tyr Asp His Gin Gly Lys Glu
381
\[ \text{Phe} \]
Val Leu Thr Pro Lys Ala Trp Asp Arg Asn Gly Gin Asp
391
Leu Thr Ala His Phe Thr Thr Ser Ile Pro Leu Lys Gly
411
Asn Val Arg Asn Leu Ser Val Lys Ile Arg Glu Cys Thr
421
Asp
Phe Gln Phe Phe
Gly Leu Ala Trp Glu Trp Trp Arg Thr Val Tyr Glu Lys
431
Thr Asp Leu Pro Leu Val Arg Lys Arg Thr Ile Ser Ile
451
Trp Gly Thr Thr Leu Tyr Pro Gin Val Glu Asp Lys Val
461
Glu Asn Asp
471

Figure 4
**INTERNATIONAL SEARCH REPORT**

**I. CLASSIFICATION OF SUBJECT MATTER** *(if several classification symbols apply, indicate all)*

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5
C07K 13/00, C12P 21/00, C12N 15/31, C07H 21/04

**II. FIELDS SEARCHED**

**Classification System | Classification Symbols**

**IPC**
- Derwent databases: WPI, WPIIL, USEPA: keywords *STREPTOCOCCUS PNEUMONIAE, PNEUMOCOCCUS, PNEUMOLYSIN HEMOLYSIN, HEMOLYSIN, CPR, COMPLEMENT BINDING REGION CPR, REACTIVE PROTEIN*

**Aust Class:** C07K 13/00, 15/04
C12N 15/31 CHEM ABS using keywords above

**III. DOCUMENTS CONSIDERED TO BE RELEVANT**

<table>
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<tr>
<th>Category*</th>
<th>Citation of Document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to Claim No</th>
</tr>
</thead>
<tbody>
<tr>
<td>PX</td>
<td><em>Infection and Immunity, Vol 57 (8) Aug 1989 p2547-2552</em> F.K. SAUNDERS et al &quot;Pneumolysin, the Thiol-Activated Toxin of Streptococcus pneumoniae, does not require a Thiol Group for In Vitro Activity&quot;</td>
<td>1, 4-5, 15-21</td>
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* Special categories of cited documents: 10
  *A* document defining the general state of the art which is not considered to be of particular relevance
  *E* earlier document but published on or after the international filing date
  *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
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**IV. CERTIFICATION**

*Date of the Actual Completion of the International Search*
6 April 1990 (05.04.90)

*Date of Mailing of this International Search Report*
12 April 1990

*International Searching Authority*
Australian Patent Office

*Signature of Authorized Officer*
R. SAWYER

Form PCT/ISA/210 (second sheet) (January 1985)