Monoclonal antibodies against *Streptococcus pneumoniae* detect epitopes on eubacterial ribosomal proteins L7/L12 and on streptococcal elongation factor Ts

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Two monoclonal antibodies (mAbs) designated 144,H-3 (IgG2a) and 218,C-5 (IgM) were produced after immunization of mice with two different heat-treated and sonicated pneumococcal strains. Western blotting, with solubilized proteins from different bacterial genera and from mammalian lymphocytes, showed that both mAbs reacted with a protein of approximately 12 kDa in all 66 strains of eubacteria examined, representing 27 different species. The 12 kDa protein was isolated by immunoaffinity chromatography. Subsequent preparative Western blotting enabled N-terminal amino acid sequence analysis by microsequencing. A high degree of amino acid sequence similarity with eubacterial ribosomal proteins L7/L12 was demonstrated. One of the mAbs (144,H-3) also cross-reacted in Western blotting with a 43 kDa protein, but only from streptococci. The 43 kDa protein carrying the common streptococcal epitope was isolated and sequenced in the N-terminal region. A high degree of amino acid sequence identity was found to elongation factor Ts from *Escherichia coli*.

Keywords: monoclonal antibodies, eubacteria, *Streptococcus pneumoniae*, ribosomal protein L7/L12, elongation factor Ts

INTRODUCTION

Infections caused by *Streptococcus pneumoniae* still remain a major cause of morbidity and mortality throughout the world, in particular among infants and elderly people. Capsular polysaccharides are essential virulence determinants and are used for classification of pneumococci into 90 types (Lund & Henrichsen, 1978; Henrichsen, 1995). This organism also has a common cell wall polysaccharide of which phosphorylcholine is the immunologically dominant epitope. When mice are immunized with pneumococci, one of the most prominent antibody responses is to this phosphorylcholine determinant (McDaniel *et al.*, 1986). This may be the reason why few reports on monoclonal antibodies

(mAbs) against pneumococcal capsular polysaccharides and protein antigens are published. This observation may also explain why only a few proteins from this Gram-positive bacterium have been characterized (Tuomanen *et al.*, 1995), even though proteins may contribute to the pathogenesis of pneumococcal infections. Furthermore, increased knowledge of common pneumococcal proteins might also be useful in the development of new vaccines.

We have immunized mice with different clinical isolates of *S. pneumoniae*. After fusion of spleen cells with myeloma cells the supernatant fluids were first screened with the strain used for immunization and purified cell wall polysaccharides. Antibodies that reacted with the bacterial strain used as immunogen, but not with the cell wall polysaccharides, were then tested by Western blotting to select those reacting with epitopes expressed on proteins. From these experiments we report two mAbs that recognize highly conserved epitopes on bacterial L7/L12 ribosomal proteins. The L7/L12 ribosomal proteins are among the most investigated components of the prokaryotic ribosomes, but there has, up

Abbreviations: DOC, sodium deoxycholate; EF-Ts, elongation factor Ts.

The EMBL accession numbers for the amino acid sequences of ribosomal L7/L12 protein from *Streptococcus pneumoniae* and *Neisseria meningitidis* reported in this paper are P80714 and P80716, respectively. The accession number for the sequence of the elongation factor from *S. pneumoniae* is P80715.

to now, only been one report on mAbs against these proteins (Sommer *et al.*, 1985). The mAbs cross-reacted with L7/L12 ribosomal proteins of other eubacteria, indicating a common conserved epitope as the reactive motif.

METHODS

Bacterial strains. The bacteria examined are described in Table 1. Most of the strains were human clinical isolates, but some were obtained from the American Type Culture Collection (ATCC). Pneumococcal strains of the different capsular types included in the 23-valent vaccine (Pneumovax; Merck, Sharp & Dohme) were typed by the capsular reaction test with rabbit antisera purchased from Statens Seruminstitut (Copenhagen, Denmark). Differentiation of types within groups was carried out at Statens Seruminstitut. Statens Seruminstitut also supplied us with *Streptococcus suis* type 8, strain 14636, and *Escherichia coli* strain U5/91. *Mycobacterium leprae* was obtained from Dr M. J. Colston (National Institute for Medical Research, London, UK). *Mycobacterium bovis*, strain Myc 14323, was received from Rijksinstituut voor Volksgezondheid en Milieuhygiëne (Bilthoven, The Netherlands). *Archaeoglobus fulgidus* strain VC-16 (Steiter, 1988) was obtained from Dr Torleif Lien (Institute of Microbiology, University of Bergen, Bergen, Norway).

Polysaccharides. Pneumococcal capsular polysaccharides were obtained from ATCC. Purified cell wall polysaccharides from *S. pneumoniae* were a gift from Dr Jørgen Henriksen (Statens Seruminstitut, Copenhagen).

Production of mAbs 144,H-3 and 218,C-5. Heat-treated (30 min at 60 °C), sonicated pneumococcal strains of capsular types 8 and 23F were used as immunogens. The type-8-derived antigen was inoculated into BALB/c mice and resulted in mAb 144,H-3. mAb 218,C-5 was generated by immunization of C57Bl mice with the antigen from the type 23F strain.

Six-week-old BALB/c and C57Bl mice were immunized with sonicated bacterial suspensions containing 50 µg protein in 0.25 ml PBS mixed with 0.25 ml Freund's incomplete adjuvant followed by a booster injection 2 weeks later with the same mixture. Four months later, and 7 d before fusion, one BALB/c mouse was injected with the above mentioned immunogen, followed by 50 µg protein in PBS on days 4 and 3 prior to fusion. Four weeks after the primary injections and 3 d prior to fusion, one C57Bl mouse was injected intravenously with 50 µg protein in PBS. All injections, except the last one to the C57Bl mouse, were given intraperitoneally. Spleen cells from the two mouse strains were fused with NSO myeloma cells by standard methods (Fazekas de St Groth & Scheidegger, 1980; Lane, 1985). Mouse splenocytes were used as feeder cells.

Hybridoma supernatant fluids were screened in an ELISA (see below) against the immunizing pneumococcal strain and against cell wall polysaccharides. Those which reacted only with sonicated and heat-treated pneumococci (30 min at 60 °C) were checked by Western blotting with bacterial proteins from the immunizing strain and from *Haemophilus influenzae*, originally used as a negative control. Hybridoma cells were cloned by limiting dilution with Hybridoma Enhancing Supplement (Sigma) instead of feeder cells. Hybridoma cells were expanded and cultured in the peritoneal cavities of Pristane-primed mice to obtain ascitic fluid. Isotyping of mAbs in hybridoma supernatant fluids was

performed in an ELISA with heat-treated (30 min at 60 °C) and sonicated pneumococci as coating antigen (see below) using a kit from Zymed Laboratories.

Protein assay. For determination of protein concentrations, aliquots of the bacteria were dissolved in 0.5 M NaOH. The Lowry method was used, with BSA as standard.

ELISA. Flat-bottomed microtitre plates (MaxiSorp, Nunc) were coated overnight at 4 °C with heat-treated (30 min at 60 °C) and sonicated pneumococci (25 µg protein ml⁻¹) in PBS with 0.02 % sodium azide, 50 µl per well. Hybridoma supernatant fluids were added in 50 µl aliquots and incubated for 2 h at 37 °C. Alkaline-phosphatase-conjugated goat anti-mouse Ig (Sigma) was used at a 1/2000 dilution in PBS containing 3 % BSA. *p*-Nitrophenyl phosphate was used as the substrate [1 mg ml⁻¹ in 10 % diethanolamine buffer (pH 9.8) containing 5 mM MgCl₂]. PBS plus Tween 20 (0.05 %) was used for the washes between each step (Kolberg *et al.*, 1992).

SDS-PAGE and immunoblotting. The bacterial suspensions in PBS were boiled for 5 min with sample buffer containing 2-mercaptoethanol (Laemmli, 1970). In the case of the mycobacteria, the bacteria were first sonicated for 20 min in iced water and then boiled as for the other samples. Samples containing 7 µg protein were placed in each well formed by a 15-tooth comb in a Bio-Rad Mini-Protean slab cell apparatus. SDS-PAGE was performed with stacking and separating gels containing 4 and 15 % (w/v) acrylamide, respectively. The separated proteins were electrotransferred to a nitrocellulose membrane (pore size 0.2 µm). To prevent non-specific binding of proteins, the membranes were incubated for 30 min in a blocking buffer consisting of 3 % BSA in PBS. The mAbs were used as hybridoma supernatant fluids diluted (range 1/10–1/1000) in blocking buffer. As secondary antibody, peroxidase-conjugated rabbit anti-mouse Ig was used (Dakopatts, dilution 1/1000). The immunostaining was performed with 3-amino-9-ethylcarbazole and hydrogen peroxide in sodium acetate buffer (pH 5.0). Rainbow protein molecular mass markers were obtained from Amersham. The protein content of the different organisms and cells, electroblotted to nitrocellulose membranes after SDS-PAGE, was controlled by protein staining with Amido black.

Cross-inhibition studies of mAb epitope specificity. Nitrocellulose membrane strips containing *S. pneumoniae* proteins separated by SDS-PAGE were blocked with 3 % BSA in PBS (see above) and then incubated for 1.5 h at room temperature with various concentrations of mAb 144,H-3 (isotype IgG2a) using hybridoma supernatant fluids diluted in blocking buffer (range 1/10–1/1000). The strips were then incubated with a 1/1000 dilution of mAb 218,C-5 (isotype IgM) followed by a biotinylated anti-mouse IgM antibody from a kit used for isotyping (Zymed Laboratories). Peroxidase-conjugated streptavidin was then added and antibody binding visualized as described above. Conversely, the inhibition of the binding of mAb 144,H-3 (IgG2a) by various concentrations of mAb 218,C-5 was examined by using a biotinylated anti-mouse IgG2a antibody. Controls included incubations of strips with the blocking buffer instead of the mAb used for inhibition. The specificity of the isotype specific antibodies was also checked in this assay.

Dot-blot assay. Heat-treated bacteria (30 min at 60 °C) from the stationary phase of growth were spotted onto nitrocellulose as previously described (Kolberg *et al.*, 1992). Some experiments were also performed with live, exponential phase pneumococci (determined by optical density measurements during growth). The primary antibodies were applied in the

form of diluted hybridoma supernatant fluids and bound mAbs were detected with peroxidase-conjugated anti-antibodies (see above).

Solubilization of bacterial proteins. Bacteria from the stationary phase of growth were harvested by centrifugation, washed three times in PBS and killed by heat-treatment at 100 °C for 5 min. The suspension was then sonicated, and nucleic acids were precipitated by addition of ethanol at 4 °C to a concentration of 20% (v/v). Proteins were precipitated by increasing the ethanol concentration to 80%. The ethanol precipitations were omitted in later experiments. The bacteria were solubilized in 0.5% (w/v) sodium deoxycholate (DOC)(Merck) in 0.05 M Tris/HCl buffer containing 2 mM EDTA (pH 8.6) for about 1 h at room temperature. Non-solubilized materials were removed by centrifugation (10 min, 4 °C, 20000 g).

Immunoaffinity purification of bacterial proteins and micro-sequencing. mAb 144,H-3 was purified from ascitic fluid by affinity chromatography on Protein A-Sepharose (Pharmacia Biotech). The mAb was then coupled to CNBr-activated Sepharose according to the recommendations by the manufacturer (Pharmacia Biotech). The gel contained about 4 mg protein ml⁻¹. The DOC extract was first run through a Sepharose 6B column to remove non-specifically binding proteins, and then through the column with immobilized mAb 144,H-3. After washing with the buffer used for application, bound proteins were eluted with 0.1 M triethylamine in distilled water (pH 11.5). The fractions were neutralized with acetic acid, dialysed against 0.01 M Tris/HCl buffer (pH 8.6) and concentrated by vacuum filtration. The eluted proteins were separated by SDS-PAGE using a comb for preparative electrophoresis and electroblotted onto a PVDF membrane (pore size 0.45 µm, Millipore). Rainbow molecular mass markers were used in the reference well of the comb. Strips were cut off and probed with mAb 144,H-3. The rest of the membrane was stained with 0.025% Coomassie brilliant blue in methanol. After destaining in 50% (v/v) methanol, bands reacting with the mAb were identified by comparison with the molecular mass markers. The strips were incubated with mAb 144,H-3 and then cut out for N-terminal amino acid micro-sequencing. Automatic Edman degradation was performed on an Applied Biosystems model 477A equipped with an on-line 120A PTH amino acid analyser.

Sequence alignment. Searches in the SWISS-PROT protein sequence databank (Bairoch & Boeckmann, 1991) and sequence alignment were done using the Wisconsin Sequence Analyses Package (Devereux *et al.*, 1984). Prediction of antigenic epitopes was determined according to Kolaskar & Tongaonkar (1990) using the European Molecular Biology Network on-line EGCG-8.0 package.

RESULTS

mAb 144,H-3 reacts with a 12 kDa eubacterial protein and also with a 43 kDa streptococcal protein in Western blotting

Dot-blotting showed that 60 different heat-treated pneumococcal strains representing all capsular types included in the 23-valent vaccine reacted with this antibody (isotype IgG2a) (data not shown). Live pneumococci did not react with mAb 144,H-3.

Thirty of the pneumococcal strains were also analysed by Western blotting against mAb 144,H-3. All showed

Fig. 1. Western blots with bacteria probed with mAb 144,H-3 (a) or 218,C-5 (b). Lanes 1 and 2, two different strains of *Streptococcus pneumoniae*; lane 3, *Streptococcus mitis*; lane 4, *Streptococcus sanguis*; lane 5, *Escherichia coli*; lane 6, *Haemophilus influenzae*; lane 7, *Micrococcus luteus*; lane 8, *Mycobacterium bovis*; lane 9, *Archaeoglobus fulgidus*.

two reactive bands, of 12 and 43 kDa (Fig. 1). The hybridoma cells had been recloned and the clones examined in Western blotting to ensure that the cultures did not contain two hybridomas producing two antibodies of the same isotype directed against different proteins. The cross-reactivity of mAb 144,H-3 with bacteria other than pneumococci was investigated by Western blotting (Fig. 1, Table 1). For all the 12 examined streptococcal species, mAb 144,H-3 reacted with both the 12 and 43 kDa proteins. In contrast, in the case of the other 15 non-streptococcal eubacterial species, both Gram-positive and Gram-negative, mAb 144,H-3 only reacted with the 12 kDa protein by Western blotting (Table 1). The two examined mycobacterial species showed much weaker reactions than the other bacteria (*M. bovis* is shown in Fig. 1, lane 8). In addition to reacting with the 12 kDa protein of *E. coli*, mAb 144,H-3 also showed a weak cross-reaction with a 30 kDa protein (Fig. 1, lane 5). Some minor differences in the mobilities of the reactive proteins were seen between the analysed organisms (Fig. 1). The only archaeobacterium examined (Table 1) did not react with mAb 144,H-3 (Fig. 1, lane 9). The epitope on the 12 kDa protein recognized by mAb 144,H-3 was expressed in all examined eubacteria, whereas the epitope on the 43 kDa protein was common only to all the tested streptococci.

Amino acid sequence similarities of eubacterial 12 kDa protein with L7/L12 ribosomal proteins and of streptococcal 43 kDa protein with elongation factor Ts

Bacterial proteins from DOC extracts were purified by immunoaffinity chromatography with the mAb bound to CNBr-Sepharose. The eluted proteins were run in preparative SDS-PAGE, and the separated proteins were electrotransferred to a PVDF membrane. In the case of *S. pneumoniae* strain 456/94 (type 6B), strips bearing the 12 and 43 kDa proteins were cut off and the N-terminal amino acid sequences of these proteins were determined. The N-terminal amino acid sequence of the

Table 1. Proteins from different organisms detected by mAb 144,H-3 in Western blots

Organisms/cells	Western blotting reactivity	
	12 kDa	43 kDa
Eubacteria		
Streptococci		
<i>S. pneumoniae</i> (n = 30)	+	+
<i>S. suis</i> type 8 SIS 14636	+	+
<i>S. mitis</i> (n = 3)	+	+
<i>S. bovis</i> 971/92	+	+
<i>S. salivarius</i> 56/93	+	+
<i>S. milleri</i> (n = 2)	+	+
<i>S. sanguis</i> (n = 3)	+	+
<i>S. pyogenes</i> ATCC 12353	+	+
<i>S. agalactiae</i> 1010/90	+	+
<i>S. equisimilis</i> C 74	+	+
<i>S. mutans</i> 604/92	+	+
<i>S. zooepidemicus</i> 44/93	+	+
Other species		
<i>Staphylococcus aureus</i> ATCC 12598	+	—*
<i>Haemophilus influenzae</i> (n = 5)	+	—
<i>Neisseria meningitidis</i> (n = 2)	+	—
<i>Escherichia coli</i> U5/41	+	—
<i>Salmonella typhimurium</i> 1406 strain 9	+	—
<i>Brucella melitensis</i> 16M	+	—
<i>Actinobacillus actinomycetemcomitans</i> ATCC 33384	+	—
<i>Listeria monocytogenes</i> 4a 52-14	+	—
<i>Micrococcus luteus</i> FH-Ba 2029	+	—
<i>Bacillus subtilis</i> ATCC 6633	+	—
<i>Bacillus stearothermophilus</i> ATCC 7953	+	—
<i>Enterococcus faecalis</i> ATCC 19433	+	—
<i>Enterococcus faecium</i> ATCC 19434	+	—
<i>Mycobacterium bovis</i> BCG	++†	—
<i>Mycobacterium leprae</i>	++†	—
Archaeobacterium		
<i>Archaeoglobus fulgidus</i> VC-16	—	—
Eukaryotic cells		
Mouse splenocytes (n = 1)	—	—
Human lymphocytes (n = 1)	—	—

* Some additional bands were seen in this region. They were also detected with mAbs against irrelevant antigens.

† Weaker reactions than for the other bacteria.

12 kDa protein from *Neisseria meningitidis* strain 44/76 (group B) was also obtained.

These Western blot analyses and the protein staining of the PVDF membrane showed two additional bands corresponding to proteins of 25 and 50 kDa, respectively. These were assumed to be light and heavy chains from leakage of mAb 144,H-3 from the affinity column.

When the sequence of the first 42 amino acids of the 12 kDa protein from *S. pneumoniae* strain 456/94 was compared to sequences in the SWISS-PROT databank, significant sequence similarity was found with bacterial ribosomal proteins. The identity in the first 42 residues

was 86% to *Bacillus subtilis* ribosomal protein B-L9, which corresponds to *E. coli* ribosomal protein L7/L12 (Fig. 2). In contrast, in the case of the 12 kDa protein of *N. meningitidis*, amino acid residues in positions 1–17 were found to be identical in only two positions with the pneumococcal 12 kDa protein (Fig. 2). However, the next three amino acids of the meningococcal protein (18–20) were identical to those of *S. pneumoniae*.

Comparison of the amino acid sequences of 16 eubacterial L7/L12 ribosomal proteins available in the SWISS-PROT databank showed conserved sequences in several regions relative to the amino acid sequence of the

12 kDa protein	
Strpn	1 5 10 15 20 25 30 35 40 A L N I E N I I A E I K E A S I L E L N D L V K A I E E E F G V T A A A P V A V A A
Nesme	? I T K - ? - L E A V G S L T ? M - - -
Bacsu	- - - - - E - - - S V - - - T V - G
43 kDa protein	
Strpn	1 5 10 15 20 25 ? E I T A K L V K E L R E K S G A G V M D A K K A L V E
Ecoli	M A - - - - S - - - - - R T - - - M - - C - - - - T -

Fig. 2. Comparison of the N-terminal amino acid sequences of *Streptococcus pneumoniae* (Strpn) 12 and 43 kDa proteins from strain 456/94 and *Neisseria meningitidis* (Nesme) 12 kDa protein from strain 44/76 with *Bacillus subtilis* (Bacsu) ribosomal protein B-L9, which corresponds to *Escherichia coli* L7/L12 (Itoh & Wittmann-Liebold, 1978) and EF-Ts from *E. coli* (Ecoli) (An et al., 1981). The proteins were isolated from DOC extracts of the bacteria by affinity chromatography on a column with mAb 144,H-3 bound to CNBr-Sepharose. The eluted proteins were separated by SDS-PAGE and electrotransferred onto a PVDF membrane. A strip of the membrane was cut off and probed with mAb 144,H-3. The rest of the membrane was stained with Coomassie brilliant blue. Strips containing the bands with the 12 and 43 kDa proteins were cut off and subjected to amino acid microsequencing. Blanks (-) indicate amino acid residues identical to those of *S. pneumoniae* at the corresponding positions.

r17_ecoli	- - S I T K D O I I R A V A A N S I V N D V V E L T S I A M E E K F G V S A A A A V A V A A - G P V E - A A - - - E E K T E F D V I	57
r17_bruab	- - M A D I A K I V D E A A E L S R D L E E K N G V S A A A P V A V A A - G G A A P A A - - - A E E K T E F D V I	61
r17_salty	- - S I T K D O I I R A V A A N S I V N D V V E L T S I A M E E K F G V S A A A P V A V A A - G G A A P A A - - - A E E K T E F D V I	57
r17_bacst	- - M A D I A K I V D E A A E L S R D L E E K N G V S A A A P V A V A A - G G A A P A A - - - A E E K T E F D V I	59
r17_bacu	- - A L N I E N I I A E I K E A S I L E L N D L V K A I E E E F G V T A A A P V A V A A - G G A A P A A - - - A E E K T E F D V I	57
r17_miclu	- - M N R E Q I I A S V K E A T I L E L N D L V K A I E E E F G V T A A A P V A V A A - G G A A P A A - - - A E E K T E F D V I	55
r17_myco	M A K L S T D E L L D A I K E M T I L E L S D F V K R F E E T I E V T A A A P V A V A A - G G A A P A A - - - A E E K T E F D V I	66
r17_myce	M S K L S T D E L L D A I K E M T I L E L S D F V K R F E E T I E V T A A A P V A V A A - G G A A P A A - - - A E E K T E F D V I	66
r17_desvm	- S S I T K E Q V V E F I A N M T V L E L S E F I K E L E E K F G V S A A A P V A V A A - G G A A P A A - - - A E E K T E F D V I	63
r17_libaf	- M S N I E S I V E K I S S L T I L Q A A E L S K R L E E K F G V S A A A P V A V A A - G G A A P A A - - - A E E K T E F D V I	61
r17_psepu	- S I T K E Q V V E F I A N M T V L E L S E F I K E L E E K F G V S A A A P V A V A A - G G A A P A A - - - A E E K T E F D V I	57
r17_cgdb	- M S N I E S I V E K I S S L T I L Q A A E L S K R L E E K F G V S A A A P V A V A A - G G A A P A A - - - A E E K T E F D V I	61
r17_strco	- A M K L S Q D D L L A A F E E M T L E L S E F V K A F E E K I D V T A A A P V A V A A - G G A A P A A - - - A E E K T E F D V I	63
r17_strgr	- A M K L S Q D D L L A A F E E M T L E L S E F V K A F E E K I D V T A A A P V A V A A - G G A A P A A - - - A E E K T E F D V I	63
r17_strat	- M A K L S Q D D L L A A F E E M T L E L S E F V K A F E E K I D V T A A A P V A V A A - G G A A P A A - - - A E E K T E F D V I	65
r17_chltr	- T T E S L E T L V D I L S R L T V L E L S E F I K E L E E K F G V S A A A P V A V A A - G G A A P A A - - - A E E K T E F D V I	63
*		
r17_ecoli	L K A - - - A G A N K V A V I K A V R G A T - G L G L K E A K D L V E S A P A A I K E G V S K D D A E A L K A L E E A G A K V E V K	120
r17_bruab	L A - - - D G A N K K V I K E V R A L T - G L G L K E A K D L V E S A P A A I K E G V S K D D A E A L K A L E E A G A K V E V K	124
r17_salty	L K A - - - A G A N K K V I K E V R A L T - G L G L K E A K D L V E S A P A A I K E G V S K D D A E A L K A L E E A G A K V E V K	120
r17_bacst	L A - A D A G A O K I V I K V V R E I T - G L G L K E A K D L V E S A P A A I K E G V S K D D A E A L K A L E E A G A K V E V K	122
r17_bacu	L A - A G A S O K I V I K V V R E I T - G L G L K E A K D L V E S A P A A I K E G V S K D D A E A L K A L E E A G A K V E V K	120
r17_miclu	L A - A S A G A K K I V I K V V R E I T - G L G L K E A K D L V E S A P A A I K E G V S K D D A E A L K A L E E A G A K V E V K	118
r17_myco	L E A - - A G D K K I V I K V V R E I T - G L G L K E A K D L V E S A P A A I K E G V S K D D A E A L K A L E E A G A K V E V K	130
r17_myce	L E A - - A G D K K I V I K V V R E I T - G L G L K E A K D L V E S A P A A I K E G V S K D D A E A L K A L E E A G A K V E V K	130
r17_desvm	L K A - - A G A N K K I V I K V V R E I T - G L G L K E A K D L V E S A P A A I K E G V S K D D A E A L K A L E E A G A K V E V K	126
r17_libaf	L E G D A K K K K I V I K V V R E I T - G L G L K E A K D L V E S A P A A I K E G V S K D D A E A L K A L E E A G A K V E V K	125
r17_psepu	L V E - - A G D K K K I V I K V V R E I T - G L G L K E A K D L V E S A P A A I K E G V S K D D A E A L K A L E E A G A K V E V K	122
r17_cgdb	L K G D D E K K K I V I K V V R E I T - G L G L K E A K D L V E S A P A A I K E G V S K D D A E A L K A L E E A G A K V E V K	126
r17_strco	L T G - - A G E K K K I V I K V V R E I T - G L G L K E A K D L V E S A P A A I K E G V S K D D A E A L K A L E E A G A K V E V K	126
r17_strgr	L T G - - A G E K K K I V I K V V R E I T - G L G L K E A K D L V E S A P A A I K E G V S K D D A E A L K A L E E A G A K V E V K	126
r17_strat	L T G - - A G D K K K I V I K V V R E I T - G L G L K E A K D L V E S A P A A I K E G V S K D D A E A L K A L E E A G A K V E V K	128
r17_chltr	L E D V P A - D K K K I G V I K V V R E I T - G L G L K E A K E N T E G L P K T V K E K T S S D A E D T V K L E A G A K A V A K	127

Fig. 3. Alignment of the amino acid sequences of L7/L12 ribosomal proteins from *Escherichia coli* (r17_ecoli), *Brucella abortus* (r17_bruab), *Salmonella typhimurium* (r17_salty), *Bacillus stearothermophilus* (r17_bacst), *Bacillus subtilis* (r17_bacsu), *Micrococcus luteus* (r17_miclu), *Mycobacterium bovis* (r17_myco), *Mycobacterium leprae* (r17_myce), *Desulfovibrio vulgaris* (r17_desvm), *Liberobacter africanus* (r17_libaf), *Pseudomonas putida* (r17_psepu), *Citrus greening* disease-associated bacterium-like organism (r17_cgdb), *Streptomyces coelicolor* (r17_strco), *Streptomyces griseus* (r17_strgr), *Streptomyces antibioticus* (r17_strat) and *Chlamydia trachomatis* (r17_chltr). The amino acid sequences are from the SWISS-PROT databank (Bairoch & Boeckmann, 1991). Proteins are numbered relative to r17_ecoli. Blanks (-) represent gaps in alignment to obtain the best identity/similarity in the sequence. The asterisk indicates Ile69.

L7/L12 ribosomal protein of *E. coli* (Fig. 3). Of these 16 eubacterial species, the first eight listed in Fig. 3 were included in the present study. All 16 sequences were analysed for antigenic epitopes using the predictive method of Kolaskar & Tongaonkar (1990). The strongest common predicted epitope for all 16 of the L7/L12 amino acid sequences was observed in the consensus sequence comprising amino acid residues 65–73 of the *E. coli* ribosomal protein L7/L12 at and around residue Ile69.

The N-terminal sequence of the 43 kDa protein of pneumococcal strain 456/94 reactive with mAb 144,H-3 is given in Fig. 2. A sequence identity of 78% with *E. coli* elongation factor Ts (EF-Ts) was found.

mAb 218,C-5 reacts with ribosomal L7/L12 proteins, but not with the same epitope as mAb 144,H-3

While characterizing mAb 144,H-3, a second mAb that bound the 12 kDa protein was found. This was designated 218,C-5 (IgM). mAb 218,C-5, unlike mAb 144,H-3, did not react with the 43 kDa pneumococcal protein with amino acid sequence identity to the *E. coli* EF-Ts (Fig. 1). However, the dot-blot patterns of the two mAbs against different Gram-positive and Gram-negative eubacteria were similar. To show that the epitopes for mAbs 144,H-3 and 218,C-5 were located on the same 12 kDa protein, Western blotting was performed with pneumococcal proteins obtained by immunoaffinity chromatography with a mAb144,H-3-CNBr-Sepharose

column. mAb 218,C-5 was also found to react with the 12 kDa protein (data not shown).

The inhibition studies using nitrocellulose strips containing separated pneumococcal proteins showed that mAb 144,H-3 did not inhibit the binding of mAb 218,C-5. A weak inhibition of the binding of mAb 144,H-3 was seen with the highest concentrations of mAb 218,C-5 (data not shown).

DISCUSSION

N-terminal amino acid sequencing of the pneumococcal 12 kDa protein reacting with mAb 144,H-3 showed a high degree of amino acid sequence identity with *Bacillus subtilis* ribosomal protein B-L9 (Fig. 2). The molecular mass of this protein is 12633 Da (Itoh & Wittmann-Liebold, 1978), which is in agreement with our estimation for the pneumococcal analogue. The *B. subtilis* B-L9 protein corresponds to *E. coli* ribosomal proteins L7/L12 (Itoh & Wittmann-Liebold, 1978). The L7/L12 proteins are essential in bacterial ribosomes for proper functioning of several elongation factors involved in protein synthesis (for a review see Liljas, 1990). Ribosomal proteins L7/L12 are encoded by the same gene and differ only by acetylation of the N-terminus of the L12 protein, which then becomes the L7 protein (Terhorst *et al.*, 1973).

Data on the nucleotide sequences of ribosomal protein genes from bacteria, or on the amino acid sequences of ribosomal proteins, are limited to a few microorganisms. Ribosomal proteins belonging to the L7/L12 family share strong amino acid sequence identity within the eubacteria (Liao & Dennis, 1994). Alignment of the complete amino acid sequences available for L7/L12 ribosomal proteins from 16 different bacterial species in the SWISS-PROT databank is shown in Fig. 3. The C-terminus contains the highly conserved regions, whereas there is less sequence conservation at the N-terminus. Our finding that the amino acid residues of the *N. meningitidis* 12 kDa protein in this region were quite different from those of the pneumococcal 12 kDa protein support this observation. Moreover, even though no sequences for the *S. pneumoniae* L7/L12 proteins are available, the fact that the three amino acids identified at amino acid positions 18–20 were identical to the pneumococcal protein suggests possible homology between the *N. meningitidis* and *S. pneumoniae* proteins.

The mAb 144,H-3 seems to detect a conserved epitope within eubacterial L7/L12 proteins. When the available amino acid sequences were analysed for antigenicity, several regions displayed strong antigenic properties. The region around residue Ile69, which in *Chlamydia trachomatis* is replaced by Leu (Fig. 3), gave the highest values, indicating that the epitope is most probably located within this helix.

The L7/L12 proteins in *M. bovis* and *M. leprae* showed much weaker Western blot reactivity than the other eubacteria. The sequence comparison showed sequence

identity in the putative epitope. It might be that these slowly growing organisms contain less ribosomal proteins than the other eubacteria.

The only examined archaeobacterium showed a lack of reactivity with the mAbs. This was expected because the archaeal equivalent L7/L12 proteins are more closely related in both amino acid sequence and size to the human P-proteins than to their *E. coli* counterparts (Casiano & Traut, 1994).

A second mAb, designated 218,C-5, also recognized the L7/L12 protein. This mAb did not cross-react with the streptococcal 43 kDa protein, indicating interaction with different epitopes. This was supported by competition studies. The epitopes are most likely closely located because the IgM antibody (218,C-5) weakly inhibited the binding of the IgG2a antibody (144,H-3).

N-terminal sequencing of the 43 kDa protein from *S. pneumoniae* reacting with mAb 144,H-3 showed 78% identity with *E. coli* EF-Ts. *E. coli* EF-Ts contains 283 amino acid residues (An *et al.*, 1981), giving a molecular mass of about 30 kDa. Western blots performed with *E. coli* showed a very faint band in the 30 kDa region that could be EF-Ts (Fig. 1). A shift in molecular mass to 43 kDa was found for the strongly reacting analogue in all examined strains of *S. pneumoniae* and other streptococci. There are no reports on amino acid sequences for eubacterial EF-Ts proteins other than *E. coli*. However, the complete amino acid sequence of *Thermus thermophilus* EF-Ts was recently reported by Blank *et al.* (1996). EF-Ts from *T. thermophilus* is considerably shorter than EF-Ts from *E. coli*, differing in size by 86 amino acids.

Our work with mAb 144,H-3 indicates similarities between ribosomal protein L7/L12 and streptococcal EF-Ts, a factor which is essential for the elongation of the polypeptide chain during protein synthesis. The two proteins recognized by mAb 144,H-3 are both components of the translational machinery and should normally be inaccessible to antibodies. This is consistent with our finding that the antigens in living pneumococci were not recognized by mAb 144,H-3 in the dot-blot assay.

It is difficult to speculate about the relationship between the conserved antigenic determinant on the L7/L12 protein and the cross-reacting epitope on streptococcal EF-Ts because we do not know the amino acid sequence of the streptococcal protein.

The mAb 144,H-3 may be a potentially valuable reagent in studying the streptococcal EF-Ts. The data from the bacteria analysed so far indicate that this epitope on EF-Ts from streptococci might represent a taxonomic marker. Interestingly, in the case of the two enterococcal species examined, mAb 144,H-3 did not react with the 43 kDa protein. The enterococci were formerly classified as streptococci, but the genus *Enterococcus* is now generally accepted (Facklam & Sahm, 1995). Our observations appear to support this new classification.

The immunological importance of ribosomal preparations of *M. bovis* and other intracellular micro-organisms such as *Brucella abortus* has been emphasized by different investigators, and the L7/L12 proteins have been identified as important antigens (Tantimavanich *et al.*, 1993; Bachrach *et al.*, 1994; Oliveira *et al.*, 1994). Ribosomal preparations have been used as vaccines in experimental animals (Normier *et al.*, 1992) and have also been examined for use as vaccines for humans (Michel *et al.*, 1978; Béné *et al.*, 1993). The mAbs described here could possibly be useful tools in the characterization and standardization of ribosomal vaccines.

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