Phillip Cash[1,2]
Evelyn Argo[1]
Linda Ford[1]
Laura Lawrie[2]
Hamish McKenzie[1]

[1]Department of Medical Microbiology
[2]Aberdeen Proteome Facility, Aberdeen, Scotland

A proteomic analysis of erythromycin resistance in *Streptococcus pneumoniae*

Streptococcus pneumoniae is a significant human pathogen which is an important cause of pneumonia and bacteraemia. Over the past few years the incidence of antibiotic resistance among clinical isolates of *S. pneumoniae* has increased. Penicillin resistance is now widespread and the frequency of isolates that are resistant to erythromycin has risen. Erythromycin resistance in *S. pneumoniae* follows two basic patterns. The MLS erythromycin-resistant phenotype is due to the enzymatic methylation of ribosomal RNA that blocks erythromycin binding to the ribosome. Alternatively, in isolates of the M phenotype, a more recently documented mechanism, resistance is associated with an active efflux process that reduces intracellular levels of erythromycin. We used two-dimensional electrophoresis to examine the proteins synthesised by erythromycin-susceptible and -resistant *S. pneumoniae*. Erythromycin-resistant *S. pneumoniae* with the M phenotype showed a significantly increased synthesis of a 38 500 Dalton (p*I* 6.27) protein compared to susceptible isolates. Peptide mass mapping was used to identify the 38 500 Dalton protein as glyceraldehyde-3-phosphate dehydrogenase (GAPDH). It was demonstrated that *S. pneumoniae* synthesised at least three forms of GAPDH that differed in their isoelectric points. The form of GAPDH possessing the most basic p*I* showed the increased synthesis in the erythromycin-resistant *S. pneumoniae* isolates. Alterations in the synthesis of GAPDH were only found for those erythromycin-resistant isolates possessing the M phenotype. *S. pneumoniae* isolates with the MLS phenotype were indistinguishable from the susceptible strains using the analytical conditions employed for the current study. The possible role of GAPDH in erythromycin resistance of *S. pneumoniae* is considered.

Keywords: *Streptococcus pneumoniae* / Peptide mass mapping / Proteome / Antibiotic resistance / Erythromycin / Glyceraldehyde-3-phosphate dehydrogenase EL 3585

1 Introduction

Streptococcus pneumoniae is a major human pathogen which is responsible for high levels of morbidity and mortality worldwide. *S. pneumoniae* is an important cause of pneumonia and bacteraemia including meningitis. Antibiotic resistance in *S. pneumoniae* has emerged as an increasing problem in the management of clinical infections, with penicillin resistance among *S. pneumoniae* isolates becoming widespread. Penicillin resistance is due to alterations in the penicillin binding proteins. Resistance to erythromycin, which is widely used as an alternative to penicillin, has increased in recent years. In England and Wales, for example, the incidence of erythromycin resistance among clinical isolates of *S. pneumoniae* increased from 2.8–8.6% between 1990 and 1995 [1]. Erythromycin acts by binding to the 23S rRNA to inhibit protein synthesis, and the resistance mechanism originally observed involves methylation of the target rRNA so that the antibiotic no longer binds [2]. The bacterial enzyme that modifies the target site in this way is encoded by the *erm* gene and in *S. pneumoniae* this is found on the transposon Tn*1545* [3], which also carries resistance to tetracycline [2]. In addition to their resistance to macrolides (*e.g.*, erythromycin) these isolates are also resistant to lincosamides (*e.g.*, clindamycin) and streptogramin B; this pattern is known as the MLS phenotype. Resistance to clindamycin in such isolates can either be constitutive (constitutively MLS resistant) or induced in the presence of erythromycin. A second pattern of erythromycin resistance in *S. pneumoniae* has recently been described in which the bacteria are resistant to macrolides such as erythromycin but remain susceptible to clindamycin even in the presence of erythromycin. These strains are designated the M phenotype and do not posses the *erm* gene or Tn*1545* and are, in the majority of cases, tetracycline-susceptible [4, 5]. Ribosomal binding of erythromycin is normal in M phenotype strains and they show no evidence of erythromycin breakdown by esterase activity. However, uptake studies of erythromycin suggest that

Correspondence: Dr. Phillip Cash, Department of Medical Microbiology and Aberdeen Proteome Facility, Polwarth Building, Foresterhill, Aberdeen AB25 2ZD, Scotland
E-mail: p.cash@abdn.ac.uk
Fax: +01224-685604

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; **MIC**, minimal inhibitory concentration

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these stains have an active efflux mechanism to remove the antibiotic [5]. The M phenotype in *Streptococcus pyogenes* is associated with the presence of the *mefA* gene that encodes a 44.2 kDa protein that is a putative efflux protein based on its partial homology to other efflux proteins [6]. A similar gene, *mefE*, has now been identified in *S. pneumoniae* strains with the M phenotype [7]. The exact role of the *mefE* product in resistance and the involvement of other bacterial genes is unknown.

To further investigate the M phenotype in *S. pneumoniae* we have used proteomics to display and characterise the proteins expressed by erythromycin-susceptible and -resistant bacteria. Proteomics has been widely used to characterise the proteins expressed by a diverse range of bacterial groups (reviewed in [8]). We have previously used this approach to define the extent of naturally occurring protein heterogeneity among clinical bacterial isolates [9–11]. Other researchers have employed proteomics to compare pathogenic variants [12–14] as well as to identify changes in bacterial gene expression induced by the extracellular environment [15–17]. Studies of antibiotic resistance mechanisms employing proteomics have been more limited. Evers *et al.* [18] reported on the action of the antibiotics sulfamethoxazole and trimethoprim on tetrahydrofolate synthesis in *H. influenzae*. In addition, protein mobility differences have been observed between rifampicin-susceptible and -resistant *Neisseria meningitidis* isolates [19]. The following report describes the application of two-dimensional electrophoresis (2-DE) to analyse the proteins expressed by erythromycin-susceptible and -resistant *S. pneumoniae* isolates. Proteins whose synthesis correlated with the M phenotype were identified using peptide mass mapping. The observed protein differences are discussed in relation to the potential mechanism of erythromycin resistance for these bacteria.

2 Material and methods

2.1 Source and characterisation of *S. pneumoniae* isolates

The *S. pneumoniae* isolates used during this study were cultured from clinical specimens submitted to the routine diagnostic laboratory in the Department of Medical Microbiology, Aberdeen University. A group of serotype 14 isolates collected during a previous survey of *S. pneumoniae* bacteraemia (submitted for publication) was studied. These included four erythromycin-resistant and six erythromycin-susceptible isolates. In addition and without previous knowledge of serotype, a further eight erythromycin-resistant *S. pneumoniae* isolates were obtained during the course of the study from a variety of clinical specimens and a further four erythromycin-susceptible isolates from positive blood cultures. Isolates were identified as *S. pneumoniae* on the basis of their characteristic α-haemolysis on blood agar and sensitivity to optochin. The bacteria were serotyped by the Scottish Pneumococcal Reference Laboratory, Stobhill Hospital, Glasgow, by coagglutination [20]. Susceptibility to erythromycin was determined by the E-test (AB Biodisk, Solna, Sweden) on Isosensitest agar (Oxoid, Basingsloke, UK) containing 5% lysed horse blood and incubated overnight at 37°C in 5% CO_2. The erythromycin-susceptible *S. pneumoniae* reference strain ATTC 49619 had a minimal inhibitory concentration (MIC) in the range 0.032–0.25 mg/L under these conditions as recommended by the E-test manufacturers. Isolates with an erythromycin MIC greater than or equal to 1 mg/L were scored as resistant. Tetracycline susceptibility was determined by Stokes' disc method [21] and inducible clindamycin resistance was detected by placing erythromycin and clindamycin disks 10 mm apart and examining plates after incubation for flattening of the clindamycin inhibition zone on the erythromycin side [5]. Isolates resistant to erythromycin, clindamycin and tetracycline were designated MLS phenotype. Isolates resistant to erythromycin only were designated M phenotype. The latter isolates were also screened for the *mefE* gene by PCR and were all confirmed as carrying the *mefE* gene.

2.2 Genotyping of *S. pneumoniae* by pulsed field gel electrophoresis (PFGE)

S. pneumoniae isolates were cultured overnight at 37°C in 5% CO_2 on two chocolate agar plates and the growth was harvested and washed in suspension buffer (10 mM Tris, 1 M NaCl). The suspension (300 μL) was mixed with 200 μL 1% InCert agarose (Anachem, Luton, UK) at 45°C and 200 μL of this mixture were added to each of two insert molds. The inserts were allowed to set at 4°C for 15 min and then incubated overnight at 37°C in 3 mL lysis buffer (10 mM Tris, 1 M NaCl, 100 mM EDTA, 0.5% polyoxyethylene 20 cetyl ether, 0.2% deoxycholate, 0.5% Sarkosyl, 1 mg/mL lysozyme). The lysis buffer was then discarded and the inserts placed in a shaking incubator for a further 24 h at 50°C in 3 mL proteinase buffer (0.5 M EDTA, pH 8.0, 0.04% Sarkosyl, 1 mg/mL proteinase K). After extensive washing in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) inserts were stored at 4°C. A piece of each insert was digested overnight at 25°C with *Sma*I (Boehringer, Mannheim, Germany) as detailed by the manufacturers. After the addition of stop solution (0.25% bromophenol blue, 0.1% SDS in TE buffer), the inserts were placed in the wells of a 1% agarose gel and run for 18 h on a Digital CHEF gel electrophoresis apparatus with an initial pulse time of 5 s and a final pulse time of 35 s. The gel was stained with 0.5% ethidium bromide and viewed by UV transillumination.

2.3 Detection of the *mefE* gene by PCR

Bacterial growth was harvested from two plates and suspended in 600 μL TE buffer and 100 μL of 0.1 M sodium deoxycholate was added. The sample was mixed and incubated at 37°C for 30 min, before extraction with 600 μL of Tris-saturated liquid phenol. The upper aqueous layer from this extraction was stored and the precipitate reextracted with 100 μL TE buffer. The combined aqueous layers were extracted with 600 μL chloroform:i-soamyl alcohol (24:1), and 50 μL of 3 M sodium acetate were added to the aqueous extract before precipitation of the DNA by addition of 900 μL absolute alcohol at –20°C. The DNA was collected by centrifugation at 11 000 × *g* for 3 min and resuspended in TE buffer at a concentration of 0.8 μg/mL. The DNA was diluted 1:20 with distilled water before use as a template in the PCR. PCR for the *mefE* gene (M phenotype) was performed as described by Sutcliffe *et al.* [22].

2.4 Preparation of bacterial proteins and analysis by 2-D electrophoresis

S. pneumoniae isolates were inoculated onto chocolate agar plates and incubated overnight at 37°C in 5% CO_2. The confluent bacterial growth produced was then scraped from the surface of three agar plates and the bacteria washed once with PBS-A and suspended in 0.5 mL of 2-D lysis buffer (0.01 M Tris-HCl, pH 7.4, 1 mM EDTA, 8 M urea, 0.05 M dithiothreitol, 10% v/v glycerol, 5% v/v Nonidet P-40, 200 μg/mL RNase A, 6% w/v pH 3.5–10 carrier ampholytes (Resolyte; Merck-BDH, Darmstadt, Germany). The bacteria were disrupted by sonication for 6 × 30 s and cooled on ice between each pulse of sonication. The suspension was clarified by centrifugation at 11 500 × *g* for 5 min and the supernatant stored at –70°C for later analysis. Soluble bacterial proteins were analysed using a small format 2-D gel system as described previously [10, 11]. Approximately 21 μg of bacterial protein were loaded to the first-dimensional gel, which consisted of 5% w/v acrylamide (0.25% w/v *N,N'*-methylenebisacrylamide), 8 M urea, 2% v/v Nonidet P-40 and 2.5% w/v pH 4–8 carrier ampholytes (Resolyte; BDH). The proteins were electrofocused for a total of 3125 Vh (500 V for 15 min and 1500 V for 2 h). The first-dimensional tube gels were equilibrated for 7 min in buffer (0.125 M Tris-HCl, pH 6.7, 2.5 mM dithiothreitol, 2.3% w/v SDS) and attached to the second-dimensional slab gel using 1% w/v agarose prepared in 0.125 M Tris-HCl, pH 6.7, 0.1% w/v SDS. The second-dimensional gels were electrophoresed at 200 V until the bromophenol blue dye present in the agarose had just migrated off the end of the gel, normally after 2 h. The proteins resolved by 2-DE were located using either colloidal Coomassie Brilliant Blue G-250 [11] or a commercially available silver stain kit (Daichii II; NBS Biologicals, Huntingdon, UK). The isoelectric points and apparent molecular weights of the proteins resolved by 2-DE were determined by coelectrophoresis with carbamylated creatine kinase (Merck-BDH) or proteins of known molecular weight, respectively [10]. The 2-D gels were digitised using a 16-bit CCD camera (1580 × 1028 pixels; LSR; AstroCam, Cambridge, UK) and transferred as 8-bit images to Phoretix 2-D software (Phoretix International, Newcastle upon Tyne, UK). The photographs of the gels presented in this report were taken from these digitised images.

2.5 Identification of proteins by peptide mass mapping

Following 2-DE, the proteins were located using colloidal Coomassie Brilliant Blue G-250 staining and the protein spots excised from the gel, washed, in-gel reduced, *S*-alkylated, and in-gel digested with trypsin (sequencing-grade modified trypsin; Promega Madison, WI, USA) as described elsewhere [23, 24]. An aliquot of the peptide extract produced by in-gel cleavage was passed through a GELoader tip which contained a small volume of POROS R2 sorbent (PerSeptive Biosystems, Framingham, MA, USA) as described [24]. The adsorbed peptides were washed extensively and then eluted in 0.5 μL of a saturated solution of α-cyano-4-hydroxycinnamic acid (Aldrich Chemical, Poole, UK) in 50% acetonitrile / 5% formic acid. The mass spectra were acquired on a PerSeptive Biosystems Voyager-DE STR MALDI-TOF mass spectrometer. The instrument was operated in the reflectron-delayed extraction mode. Spectra were internally calibrated using trypsin auto-digestion products. A non-redundant protein sequence database maintained and updated at EMBL and The European Bioinformatics Institute was used for all database searches using the PeptideSearch software package. At the time of use this database contained more than 257 000 entries. The search parameters used were as follows: cysteine as *S*-carbamidomethyl-derivative; maximum allowed peptide mass error of 50 ppm; more than five peptide mass hits required for a protein match. No restriction was placed on either the isoelectric point or species of origin of the protein. A protein mass range between 0 and 150 kDa was allowed.

3 Results

3.1 Characterisation of erythromycin-resistant isolates of *S. pneumoniae*

We have previously observed a high incidence of M phenotype resistance among serotype 14 *S. pneumoniae* isolates collected in Grampian, Scotland, during a study of

bacteraemia between 1994 and 1996 (submitted for publication). PFGE enabled serotype 14 isolates to be divided into one of two genetic clusters (designated I and II) with similar, although not identical patterns (Fig. 1). A similar differentiation of the bacterial isolates was also found using restriction enzyme analysis with other restriction enzymes (unpublished data). All erythromycin-resistant isolates were in group I and had MICs in the range 12–24 mg/L erythromycin. Two serotype 14 isolates in this cluster were erythromycin-susceptible (MIC < 1 μg/mL). Resistant isolates of the MLS phenotype were of various serotypes (Table 1).

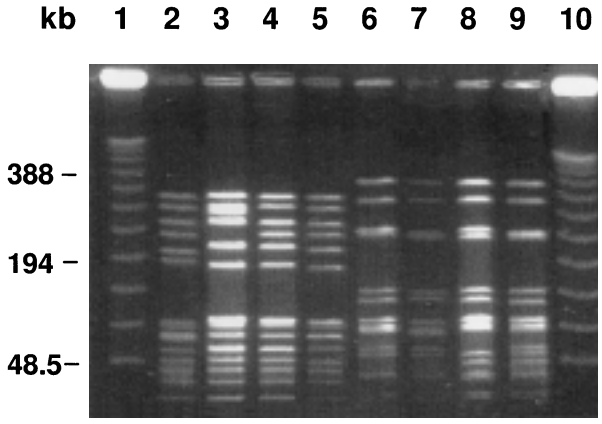

Figure 1. PFGE of *SmaI* digests of erythromycin-resistant (lanes 2, 3 and 4) and erythromycin-susceptible (lanes 5–9) serotype 14 *S. pneumoniae* isolates. Isolates in lanes 2–5 are designated group I genotype and those in lanes 6–9 as group II genotype. Lanes 1 and 10 contain molecular weight markers.

3.2 Characterisation of *S. pneumoniae* proteins by 2-D electrophoresis

We have previously used nonionic detergent to lyse Gram-negative bacteria to prepare soluble proteins for analysis by 2-DE [8, 11, 19]. For these bacteria efficient disruption of the cells was achieved following a short incubation of the bacterial suspension in the presence of NP-40 on ice. However, the same conditions of cell lysis failed to disrupt *S. pneumoniae* and it was necessary to use sonication in the presence of NP-40 as described in Section 2.4. The soluble bacterial proteins prepared from *S. pneumoniae* by this method were analysed using a

Table 1. Properties of *S. pneumoniae* isolates

Isolate number	Serotype	Genotypic group[a)]	Antibiotic resistance			Phenotype[b)]	*p38.5*[c)]
			Erythromycin	Clindamycin	Tetracycline		
346R	14	I	R	S	S	M	High
306R	14	I	R	S	S	M	High
359R	14	I	R	S	S	M	High
387R	14	I	R	S	S	M	High
E14R	14	I	R	S	S	M	N.D.
E20R	14	I	R	S	S	M	High
E18R	14.	I	R	S	S	M	High
E27R	14.	I	R	S	S	M	High
E30R	14.	I	R	S	S	M	High
B04S	14	I	S	S	S	S	Low
437S	14	I	S	S	S	S	Low
183S	14	II	S	S	S	S	Low
184S	14	II	S	S	S	S	Low
B01S	14	II	S	S	S	S	Low
B03S	14	II	S	S	S	S	Low
B09S	14	II	S	S	S	S	Low
441S	4	N.A.	S	S	S	S	Low
E01R	15	N.A.	R	R	R	MLS	Low
E15R	6	N.A.	R	R	R	MLS	Low
E22R	23	N.A.	R	R	R	MLS	Low

a) Examples of the patterns are shown in Fig. 1
N.A., not applicable
N.D., no data
b) Erythromycin resistance phenotype – see text for description of the M and MLS phenotypes
S, susceptible
c) Expression levels of *p38.5*

small format 2-D gel system. When colloidal Coomassie Brilliant Blue G-250 staining was used for protein detection, up to 200 proteins were detected within the p*I* range of 4–7 and molecular weight range of 15 000–110 000 (Fig. 2A). Silver staining increased the number of detectable bacterial proteins to approximately 360 within the same p*I* and molecular weight range (Fig. 2B). The majority of the proteins resolved under these conditions had isoelectric points < 6.2, based on the carbamylated marker proteins, which contrasted with the broader distribution of isoelectric points determined for the soluble proteins prepared from Gram-negative bacteria analysed under the same electrophoretic conditions [8, 10, 11, 19].

3.3 Analysis of protein synthesis in antibiotic-resistant *S. pneumoniae* isolates

The soluble proteins synthesised by the *S. pneumoniae* isolates were characterised by 2-DE as described in Section 2.4. For some isolates, protein synthesis was determined for a number of independent preparations and in all cases consistent protein profiles were obtained in these replicate analyses. Representative 2-D protein profiles are shown in Fig. 3 for erythromycin-susceptible and M phenotype-resistant *S. pneumoniae* isolates. A consistent difference was found between the protein profiles of erythromycin-susceptible and -resistant isolates in the synthesis of an abundant protein of 38 000 Daltons (p*I* 6.27), designated *p38.5* (Fig. 3B). In some instances a minor protein migrated in the equivalent position to *p38.5* for erythromycin-susceptible *S. pneumoniae* isolates. This was most clearly demonstrated when silver staining was used to detect the proteins (Fig. 4). This suggested that *p38.5* represented the induced synthesis of a normally synthesised bacterial protein rather than the *de novo* synthesis of a novel protein.

The increased synthesis of *p38.5* was consistently associated with erythromycin-resistant *S. pneumoniae* isolates exhibiting the M phenotype (Table 1). All of the isolates

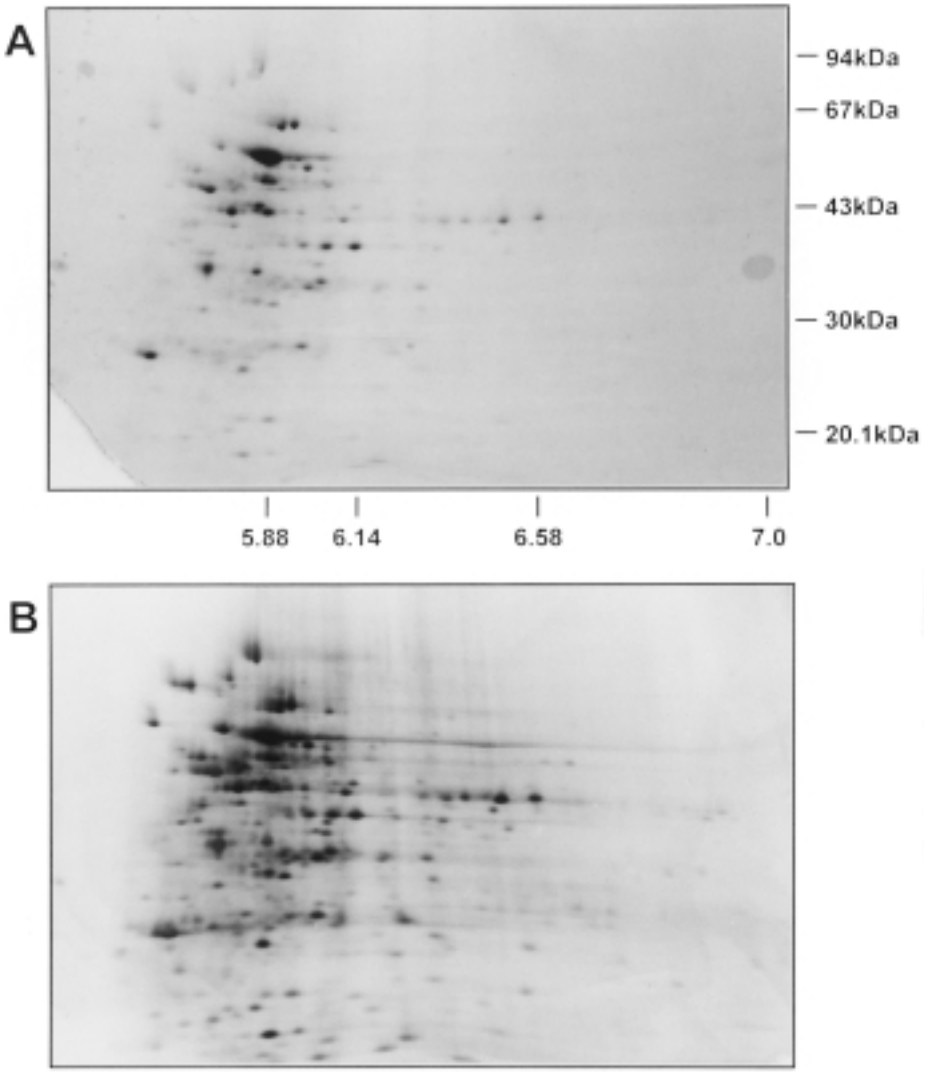

Figure 2. Analysis of soluble bacterial proteins prepared from an erythromycin-susceptible isolate of *S. pneumoniae* by 2-DE. Soluble proteins were prepared and analysed by 2-DE as described in Section 2.4. (A) Proteins detected using colloidal Coomassie Brilliant Blue G-250 staining; the locations of molecular weight markers and carbamylated protein markers, electrophoresed on parallel gels are shown. (B) Proteins detected using silver stain.

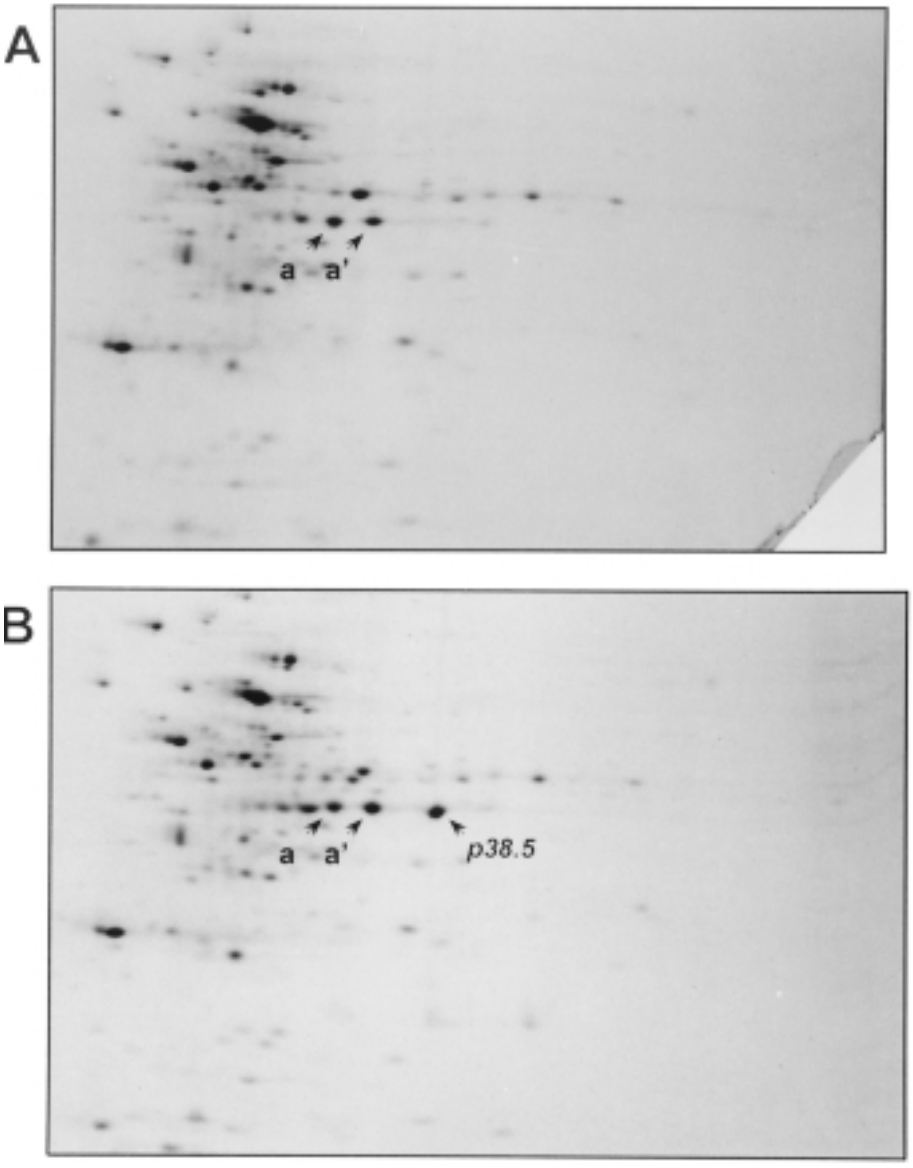

Figure 3. Analysis of soluble bacterial proteins prepared from erythromycin-susceptible and M phenotype-resistant isolates of *S. pneumoniae* by 2-DE. Soluble proteins were prepared and analysed by 2-DE as described in Section 2.4. The proteins were detected using colloidal Coomassie Brilliant Blue G-250 staining. (A) Soluble proteins prepared from erythromycin-susceptible *S. pneumoniae* isolate. (B) Soluble proteins prepared from erythromycin-resistant *S. pneumoniae* (M phenotype) isolate. The gels are oriented with the acidic proteins to the left of each panel and the high molecular weight proteins at the top.

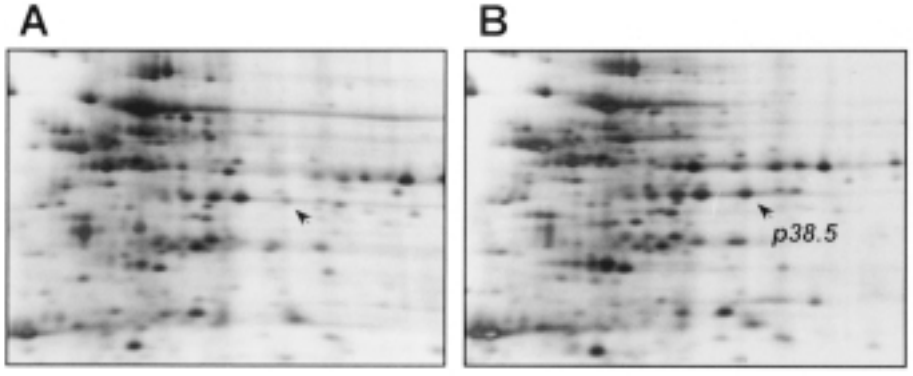

Figure 4. Analysis of soluble bacterial proteins prepared from erythromycin-susceptible and -resistant isolates of *S. pneumoniae* by 2-DE. Soluble proteins were prepared and analysed by 2-DE as described in Section 2.4. The proteins were detected using silver staining. The panels show enlarged regions of the gel in the region of *p38.5*. (A) Soluble proteins prepared from erythromycin-susceptible *S. pneumoniae* isolate; arrow indicates protein that comigrated with *p38.5*. (B) Soluble proteins prepared from erythromycin-resistant *S. pneumoniae* (M phenotype) isolate. The gels are oriented with the acidic proteins to the left of each panel and the high molecular weight proteins at the top.

examined showing the M phenotype were classified as serotype 14 and, more specifically, formed a distinct genotypic cluster within this serotype as defined using PFGE. Support for the association of *p38.5* expression with the M phenotype, rather than a specific genotype, was provided by the analysis of the erythromycin-susceptible isolates 437S and B04S. These serotype 14 isolates belonged to the same genotypic cluster as the erythromycin-resistant M phenotype isolates but showed only a minimal level of *p38.5* synthesis. The soluble proteins synthesised by three isolates exhibiting the MLS phenotype were also characterised by 2-DE. These isolates showed no induced *p38.5* synthesis. Under the analytical conditions used for the current study they were indistinguishable from the susceptible *S. pneumoniae* isolates examined to date (data not shown).

3.4 Identification of *p38.5* by peptide mass mapping

Peptide mass mapping is increasingly used for the identification of proteins resolved by 2-DE and is particularly useful where complete or extensive genome sequence information is available as in the case of many bacteria. This approach was used to identify *p38.5* following the procedure described in Section 2.5. The peptide mass map obtained following in-gel digestion of *p38.5* is shown in Fig. 5. The PeptideSearch program identified *p38.5* as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) through homology to the corresponding protein from *S. equisimilis* and *S. pyogenes*. Table 2 shows the peptides of *p38.5* that were matched to GAPDH along with the peptide mass error, the position of the peptide in the sequence and the corresponding sequence for each peptide. The minor protein identified in susceptible *S. pneumoniae* isolates that comigrated with *p38.5* was also identified using peptide mass mapping. This protein also matched, on the basis of homology, GAPDH (data not shown). Two proteins (labeled as a and a' in Fig. 3) were synthesised by both erythromycin-susceptible and -resistant *S. pneumoniae* isolates. These proteins were of the same molecular weight as *p38.5* but differed in their isoelectric points. When proteins prepared from susceptible and resistant isolates were coelectrophoresed on the same gel it was observed that, on occasion, a and a' from the resistant isolate had a slightly lower apparent molecular weight compared to the corresponding proteins of the susceptible isolate (data not shown). Peptide mass mapping was used to identify proteins a and a' from both erythromycin-susceptible and -resistant isolates. Both of these proteins were homologous to GAPDH (data not shown).

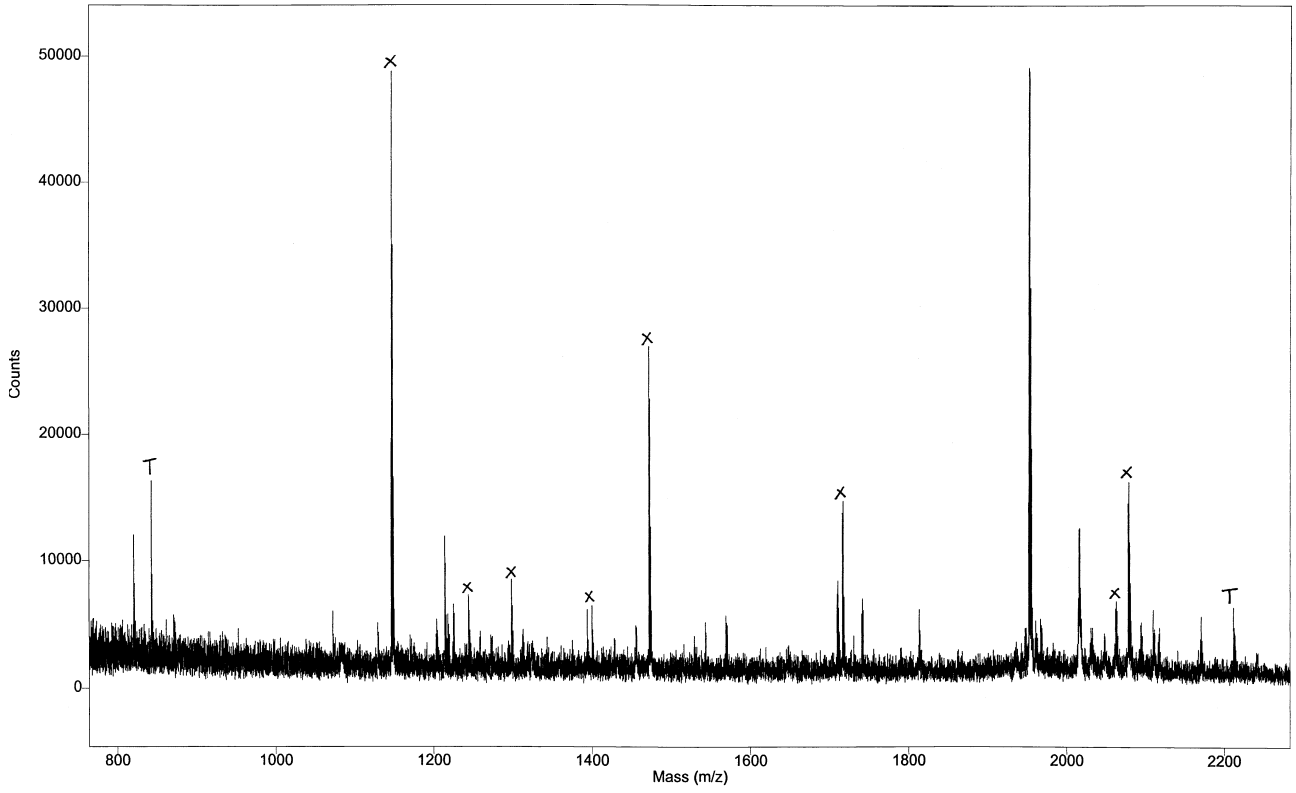

Figure 5. Peptide mass map of *p38.5* synthesised by an erythromycin-resistant (M phenotype) *S. pneumoniae* isolate.

Table 2. Peptide assignment for *p38.5*

Experimental mass	Matching mass	Mass difference (ppm)	Position	Peptide sequence
1145.6885	1145.6538	–30.3	4–14	VGINGFGRIGR
1145.6885	1145.6789	–8.4	1–11	VVKVGINGFGR
1243.7062	1243.6641	–33.9	20–30	IQNVEGVEVTR
1297.7996	1297.7474	–40.3	116–128	KVVITAPGGNDVK
1399.8167	1399.7652	–36.8	19–30	RIQNVEGVEVTR
1471.9445	1471.8855	–40.1	1–14	VVKVGINGFGRIGR
1715.8923	1715.8235	–40.1	46–60	YDTTQGRFDGTVEVK
2061.0396	2060.9746	–31.6	309–325	VVSWYDNEMSYTAQLVR
2077.0395	2076.9695	–33.7	309–325[a)]	VVSWYDNEMSYTAQLVR

a) Methionine oxidised

4 Discussion

4.1 Association of *p38.5* synthesis with erythromycin resistance

The data presented demonstrated a positive correlation between the synthesis of a 38 500 Da protein (designated *p38.5*) and the M phenotype of erythromycin resistance in *S. pneumoniae*. The association between *p38.5* synthesis and antibiotic resistance was based on the characterisation of clinical isolates of *S. pneumoniae*. Since the clinical isolates were not a genetically homogeneous population, there were likely to be protein differences between isolates which were unrelated to antibiotic resistance. Differences in minor bacterial proteins were observed between some susceptible and resistant isolates, but these were not consistently linked with the M phenotype. These minor proteins require further characterisation to assess their significance in the context of erythromycin resistance. Increased *p38.5* synthesis correlated only with the M phenotype and all such isolates were serotype 14 and fell within a single genotypic group demonstrated using PFGE. The strongest evidence for the association of *p38.5* synthesis and the M phenotype erythromycin resistance is the significantly reduced *p38.5* synthesis in isolate numbers 437S and B04S, which were a serotype 14 erythromycin-susceptible isolate within the same genotypic cluster as the resistant isolates. Nevertheless, the reliability of the association will be further established as more clinical isolates are characterised with an increased diversity of capsular antigens and genotypes. Increased *p38.5* synthesis was not apparently linked to erythromycin resistance *per se* and was specifically observed only for *S. pneumoniae* isolates with the M phenotype. Three erythromycin-resistant *S. pneumoniae* isolates possessing the MLS phenotype showed *p38.5* synthesis indistinguishable from susceptible isolates. However, these data should be interpreted with caution, since isolates of the MLS phenotype were of different serotypes from the M phenotype isolates and the extent of protein differences between different capsular types of *S. pneumoniae* is unknown.

4.2 Identification of *p38.5* as GAPDH and its proposed involvement in erythromycin resistance

Peptide mass mapping identified *p38.5* as GAPDH. The identification of *p38.5* was based on the homology of its peptide mass profile with the profiles predicted from the GAPDH sequences of *S. equisimilis* and *S. pyogenes*. Several of the major peptides in Fig. 5 were not assigned to the sequences of GAPDH from *S. equisimilus* and *S. pyogenes*, nor could they be identified as trypsin autodigestion products or as keratin contaminants. The *p38.5* peptides that failed to produce matches against the sequenced proteins may have been derived from regions of GAPDH with low homology to these bacteria.

Two additional forms of GAPDH were synthesised by both erythromycin-susceptible and -resistant isolates; these proteins are labeled a and a' in Fig. 2. The synthesis of these two forms of GAPDH was independent of the antibiotic-resistant phenotype of the bacteria. In addition, a minor protein synthesised by susceptible isolates comigrated with *p38.5* and was shown to be GAPDH. These data were consistent with a post-translational modification, for example phosphorylation, of GAPDH which led to the alternative forms of the enzyme that differed in their isoelectric points. Due to the low sequence coverage (*ca.* 27%) of GAPDH in the peptide mass maps it was not possible to identify post-translational modifications using this approach. These data supported a model in which *p38.5* (*i.e.*, the form of GAPDH with the most basic iso-

electric point) was present in the soluble protein fraction at low levels in susceptible *S. pneumoniae* isolates but increased in abundance in those isolates showing the M phenotype.

The *mefE* gene is required for the M phenotype resistance and the product of this gene is thought to be directly involved in membrane transport [7]. However, the predicted product of this gene (molecular weight 43793 and p*I* 8.88) was not resolved under the electrophoresis conditions used in the present study and it is certainly quite distinct from *p38.5*, an observation supported by the data derived from peptide mass mapping. Broad range (pH 3–10) immobilised pH gradient gels were used in the first-dimensional separation in order to look for the *mefE* gene product. Silver staining was used for protein detection. Under these conditions no protein was located with the expected electrophoretic properties of the *mefE* gene product identified. The inability of detecting the *mefE* gene product may be due either to its being synthesised at levels too low for detection or the known poor solubilisation of membrane proteins restricting its entry to the first-dimensional gel.

The possible role of GAPDH in erythromycin resistance is intriguing. The normal function of GAPDH in cellular metabolism is the conversion of glyceraldehyde-3-phosphate to 1, 3-diphosphoglycerate with the concomitant production of NADH. This is an important step in glycolysis and its possible relationship to M phenotype erythromycin resistance is not immediately obvious and may be a chance association. Since the M phenotype erythromycin resistance is mediated by an active efflux mechanism [5], it is possible that GAPDH is upregulated to help provide energy, *via* NADH, for the process. However, there is also evidence that GAPDH is not necessarily limited to a basic metabolic function within the cell. GAPDH is a major surface protein surface of *S. pyogenes* [25] and has been shown to act as an ADP-ribosylating protein [26]. Other workers have identified a plasmin receptor on *S. pyogenes* with significant homology to GAPDH [27]. Brefeldin A inhibits membrane transport in eukaryotic cells and also stimulates ADP-ribosylation of GAPDH [28]. Although not proving that GADPH is directly involved in membrane transport, the relationship between these two actions of brefeldin A is interesting in the context of the present study.

Whilst the significance of GAPDH in M phenotype erythromycin resistance in *S. pneumoniae* remains to be established, this study has demonstrated the potential of proteomics to provide unexpected insights into molecular mechanisms by virtue of its ability to study protein expression in its entirety without making advance judgements of the potential importance of different components. It appears that *p38.5* represents either a change in the level of gene expression or a post-translational modification of an existing gene product which would not have been readily identified by the application of conventional nucleic-acid-based technologies. Further studies are required to fully explore the usefulness of this approach to the study of antibiotic resistance, or indeed, other bacterial virulence factors.

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