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Differential Protein Expression in Phenotypic Variants of *Streptococcus pneumoniae*

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Streptococcus pneumoniae **undergoes spontaneous phase variation resulting in opaque and transparent colony forms. Differences in colony opacity correlate with differences in virulence: the transparent variants are more capable of colonizing the nasopharynx, whereas the opaque variants show increased virulence during systemic infections. To gain insight into the pathogenesis of pneumococcal disease at the molecular level, protein expression patterns of the phenotypic variants of two pneumococcal strains were compared by high-resolution two-dimensional protein electrophoresis. In comparison with transparent variants, the opaque variants reduced the expression of two proteins and overexpressed one protein. The proteins were identified by mass spectrometric analysis. The protein overexpressed in the opaque phenotype revealed significant homology to elongation factor Ts of *Helicobacter pylori*. One of the two proteins that were underexpressed in the opaque variants revealed significant homology to the proteinase maturation protein PrtM of *Lactocobacillus paracasei*, a member of the family of peptidyl-prolyl *cis*/*trans* isomerases. A consensus lipoprotein signal sequence suggests that the putative proteinase maturation protein A, designated PpmA, is located at the surface of the pneumococcus and may play a role in the maturation of surface or secreted proteins. The second underexpressed protein was identified as pyruvate oxidase, SpxB. The lower SpxB expression in opaque variants most probably explains the reduced production of hydrogen peroxide, a reaction product of SpxB, in this variant. Since a *spxB*-defective pneumococcal mutant has decreased ability to colonize the nasopharynx (B. Spellerberg, D. R. Cundell, J. Sandros, B. J. Pearce, I. Idanpaan-Heikkila, C. Rosenow, and H. R. Masure, 1996. Mol. Microbiol. 19:803–813, 1996), our data suggest that SpxB plays an important role in enhancing the ability of transparent variants to efficiently colonize the nasopharynx.**

A critical process in the pathogenesis of infections caused by *Streptococcus pneumoniae* is the ability of the pathogen to adapt to various ecological niches in the human host. The pneumococcus colonizes the human nasopharynx and may spread locally to cause upper and lower respiratory tract infection. In some cases, pneumococci are able to enter the bloodstream and cause bacteremia or cross the blood-brain barrier and cause meningitis.

As is the case for other respiratory tract pathogens that frequently cause invasive infection, the ability to survive in these different host environments requires the regulation of the synthesis of key surface structures (10, 31). In *S. pneumoniae*, the reversible expression or phase variation in structures can be detected as spontaneous, reversible changes in colony morphology. Differences in surface molecules affect the arrangement of organisms within a colony, resulting in a change in colony appearance (32). The frequency of switching in colony morphology is highly variable from isolate to isolate and appears to be independent of in vitro growth conditions including pH, temperature, and osmolarity (32). Genetic analysis has demonstrated that a stem-loop-forming repetitive element, BOX A-C, located upstream of the *glpF* gene, increases the variation in opacity (23).

Differences in colony morphology correlate with differences in in vitro and in vivo characteristics. In an infant-rat model of nasopharyngeal carriage, only the transparent phenotype is able to establish dense and stable colonization of the mucosal surface of the nasopharynx (32). This can be explained by the enhanced binding of transparent pneumococci to buccal epithelial cells and their glycoconjugate receptors when compared to opaque pneumococci (5). Similarly, the adherence of transparent pneumococci to cytokine-stimulated human type II lung cells and human vascular endothelial cells is enhanced, as well as the receptors (*N*-acetyl-D-glucosamine and platelet-activating factor [PAF] receptor) that appear on these cells after cytokine stimulation (5). On the other hand, the opaque variant is more virulent in an animal model of systemic infection following intraperitoneal inoculation of adult mice (13). The higher virulence of the opaque variant in an in vivo model of sepsis correlates with decreased opsonophagocytic killing of opaque pneumococci in the in vitro phagocytosis assay (12). Finally, the transparent variants have an increased capacity to cross the blood-brain barrier (20). The higher binding affinity of transparent pneumococci to the PAF receptor on microvascular endothelial cells is suggested to result in increased transcytosis of bacteria across these cells.

To gain insight in the pathogenesis of pneumococcal disease at a molecular level, the relationship between several previously identified cell surface structures and opacity variation has been examined. The opaque phenotype is associated with larger amounts of capsular polysaccharide than is the transparent phenotype (12, 13). In contrast, the transparent phenotype produces increased amounts of teichoic acid, which con-

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tains phosphorylcholine (13, 30). Phosphorylcholine forms an anchor for at least eight choline-binding proteins (21). This structure is also part of PAF and is suggested to be important in the attachment to cytokine-activated human cells via the PAF receptor by structural mimicry (5). Differences in the amount of phosphorylcholine might explain the switch between adherent and nonadherent phenotypes. Phenotypic variation also correlates with differential expression of cell surface proteins, including three choline-binding proteins, LytA, PspA and CbpA. Opaque variants undergo spontaneous lysis more slowly as a result of the decreased expression of the major amidase, LytA (33). In contrast to LytA, PspA is expressed in greater amounts in the opaque variant (13). PspA inhibits complement activation, thereby reducing the effectiveness of complement receptor-mediated pathways of clearance (27). In addition, PspA binds lactoferrin, an iron-sequestering glycoprotein that predominates in mucosal secretions, and may function in iron acquisition at mucosal surfaces (9). The differential expression of CbpA is similar to that of LytA, such that transparent variants express increased amounts of this protein (21). CbpA mediates adherence to cytokine-activated human lung epithelial and endothelial cells, is involved in invasion through microvascular endothelial cells, and participates in pneumococcal colonization of the nasopharynx (20, 21). The expression levels of LytA, PspA, and CbpA are unlikely to determine colony morphology directly, since mutants lacking each of the encoding genes still undergo variation in colony morphology (13, 21, 30).

The purpose of this study was to identify additional proteins that are differentially expressed in the phenotypic variants of *S. pneumoniae* to gain insight into the molecular changes that occur during phase variation. This information will contribute to an improved understanding of the molecular adaptation of phenotypic variants of pneumococci that result in changes in virulence and colony morphology. This paper reports on a comparison of expression patterns of the phenotypic variants of two pneumococcal strains by high-resolution two-dimensional protein electrophoresis and the identification of differentially expressed proteins by mass spectrometry.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and growth medium. Bacteria were removed from storage at −70°C and cultured at 37°C on Columbia agar supplemented with 5% defibrinated sheep blood (Oxoid, Basingstoke, United Kingdom) in an atmosphere of increased CO_2. Bacterial colonies were inoculated in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) supplemented with 0.5% yeast extract (Difco Laboratories) (THY broth) and grown at 37°C. Broth cultures were plated onto THY agar plates (15 g of agar per liter) impregnated with 80 U of catalase (Worthington Biochemical, Freehold, N.J.) per cm^2 and incubated at 37°C in an atmosphere of increased CO_2 that was provided in a candle extinction jar. Colony morphology of all cultures was determined as described previously (32). The pneumococcal strains used in this study are the opaque and transparent variants of type 9V clinical isolate p10 (32) and the opaque and transparent variants of type 6B clinical isolate p314 (12). *S. pneumoniae* D39 (2) and a *spxB*-defective mutant (D39; *spxB*::pHRM104, *phoA*$^+$ [26]) were used as control strains used in the hydrogen peroxide assay. A *ppmA*-defective mutant (D39; *ppmA*::*ermAM* (16a) was used as a control strain used for Western blot analysis.

Protein sample preparation. The bacteria were cultured overnight in 10 ml of THY broth and subsequently to logarithmic growth phase (optical density at 550 nm = 0.3) in 250 ml of THY broth. This culture was harvested by centrifugation (1,500 × *g* for 15 min) and washed twice with 250 ml of phosphate-buffered saline (pH 7.5) and once with 10 ml of Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA). The cells were disrupted by ultrasonic treatment (15 min with a microtip in a model 250 sonifier [Branson Ultrasonics, Danbury, Conn.]) while being held at 5°C. Protein concentrations were determined by the method of Bradford (3).

Two-dimensional protein gel electrophoresis, staining, and computerized comparison of the protein profiles. Isoelectric focusing (pI 4 to 7) was performed with a Multiphor II electrophoresis unit and Immobiline DryStrips (Pharmacia Biotech, Uppsala, Sweden) as recommended by the manufacturers and with the modifications described by Rabilloud et al. (18). The proteins were separated in the second dimension by gradient (12 to 20% polyacrylamide) polyacrylamide gel electrophoresis. Bacterial lysates containing 300 μg of protein were analyzed in the individual experiments. Polyacrylamide gels were stained with Coomassie brilliant blue (CBB) (24). The software program PD Quest (PDI, New York, N.Y.) was used for the computerized analysis of two-dimensional protein profiles. The relative amount of a protein, represented in parts per million (ppm), was determined by dividing the spot quantity by the total density of all proteins in the gel, thereby normalizing the amount of a single protein to the total amount of protein loaded. Significant differences in protein expression levels in the phenotypic variants were determined by the Mann-Whitney test with a set value of $P \leq 0.05$.

Purification, tryptic digest and mass spectrometric analysis of the proteins. The protein gel spots of interest were excised from the gel. The gel fragments were sliced thinly and washed twice for 15 min in 5% trichloroacetic acid (CCl_3COOH [Merck, Darmstadt, Germany]) and three times in distilled water. The gel fragments were equilibrated in sample buffer (pH 6.8) (0.1% sodium dodecyl sulfate [SDS], 10% glycerol, 50 mM dithiothreitol, 12 mM Tris-HCl, 0.01% bromophenol blue indicator [Merck]) for 1 h at room temperature. The proteins were concentrated by an agarose electrophoresis (1% agarose type VIII [Sigma, St. Louis, Mo.]) method as described by Rider et al. (19) and Gevaert et al. (7) on a model 150-A gel electrophoresis cell (Bio-Rad Laboratories, Richmond, Calif.) with Pasteur pipettes. The agarose gel was stained with carconcarboxylic acid (Sigma), and the proteins were excised from the gel. The agarose fragments were washed with distilled water, and resuspended in 18 μl of digestion buffer (pH 8.0) (50 mM NH_4HCO_3, 5 mM $CaCl_2$). The agarose was melted at 85°C for 1 min. After it was cooled to 37°C, 0.05 μg of trypsin (trypsin modified sequencing grade [Promega, Madison, Wis.]) per ml was added for at least 15 h at 37°C to digest the proteins. Trypsin was inactivated by adding 1 μl of 10% trifluoroacetic acid CF_3COOH [Merck]). The tryptic digests were analyzed using a reversed-phase microcapillary column-switching high-pressure liquid chromatography system (16, 28). Peptide sequencing was performed on an LCQ quadrupole ion trap mass spectrometer (Finnigan MAT, San Jose, Calif.). Tandem mass spectrometric data were collected in data-dependent scan mode for sequence information on single tryptic digest products. With Peptide Search (14), the deduced (partial) amino acid sequences were analyzed for matching sequences in all possible translation products of the most current version of the unfinished pneumococcal genome released by The Institute for Genomic Research (TIGR) (http://www.tigr.org/data/s_pneumoniae/) to identify the proteins. With the BLAST algorithm (1), putative pneumococcal proteins were analyzed for similarity to sequences deposited in the November 1999 version of the nonredundant protein database at the National Center for Biotechnology Information (Washington, D.C.).

Hydrogen peroxide assay. Hydrogen peroxide production by pneumococci was determined by the method of Pick and Keisari (17) and modified by Duane et al. (6). Bacteria were grown to mid-log phase, washed, and then grown in 250 μl of brain heart infusion broth (BBL Microbiology Systems, Cockeysville, Md.) in 96-well plates. Negative control wells contained 1,000 U of catalase per ml. After 1 h of incubation at 37°C, the cultures were harvested and centrifuged at 10,000 × *g* for 2 min and the supernatant was filtered through 0.2-mm-pore-size filters. Phenol red and horseradish peroxidase were added to the assay buffer (5.0 mM K_2HPO_4, 1.0 mM KH_2PO_4, 140 mM NaCl, 0.5 mM glucose [pH 7.4]) at a final concentration of 0.46 mM and 0.046 U/ml, respectively, and the buffer was immediately used in the assay. Duplicate 250-μl aliquots of filtered supernatant were then mixed with 1.05 ml of assay mixture and incubated for 30 min at 37°C. The reactions were stopped by the addition of 5.0 μl of 1.0 N NaOH. The optical density was recorded at 610 nm. Concentrations were calculated from a standard curve generated for each assay by adding known dilutions of 30% H_2O_2 to 250-μl aliquots of control supernatant. Control supernatant was heated to 100°C for 20 min before addition of H_2O_2 to eliminate catalase activity. Finally, the optical density of the culture as well as the number of CFU per milliliter was determined.

Western blot analysis. One-dimensional SDS polyacrylamide gel electrophoresis was carried out in the Bio-Rad minigel system with 13% polyacrylamide gels. Bacterial lysates (0.5 μg) were dissolved in sample buffer (10 mM Tris-HCl, 1 mM EDTA, 1% SDS, 10 mM dithiothreitol, 10% glycerol, 0.01% bromophenol blue), boiled for 5 min, and subjected to electrophoresis (24). The proteins in the gel were transferred to Immobilon-P membranes (Millipore Corp., Bedford, Mass.) as described by Sambrook et al. (24). The membranes were washed twice for 10 min each with Tris-HCl-buffered saline (pH 7.5) supplemented with 0.05% Tween 20 (TBSt). The membranes were incubated with 2% bovine serum albumin for 1 h at room temperature, washed twice for 10 min each with TBSt, and incubated with 1:10,000-diluted anti-PpmA rabbit serum (Overweg et al., submitted) for at least 2 h with constant stirring. After the washing step, 1:4,000-diluted anti-rabbit immunoglobulin-alkaline phosphatase conjugate (Sigma) was added. After 1 h, the membranes were washed in TBSt and then in alkaline phosphatase buffer (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, 5 mM $MgCl_2$). The blots were incubated with 0.33 mg of nitroblue tetrazolium (Sigma) per ml and 0.17 mg of 5-bromo-4-chloro-3-indolylphosphate (Sigma) per ml in alkaline phosphatase buffer in the dark. The staining reaction was stopped with distilled water.

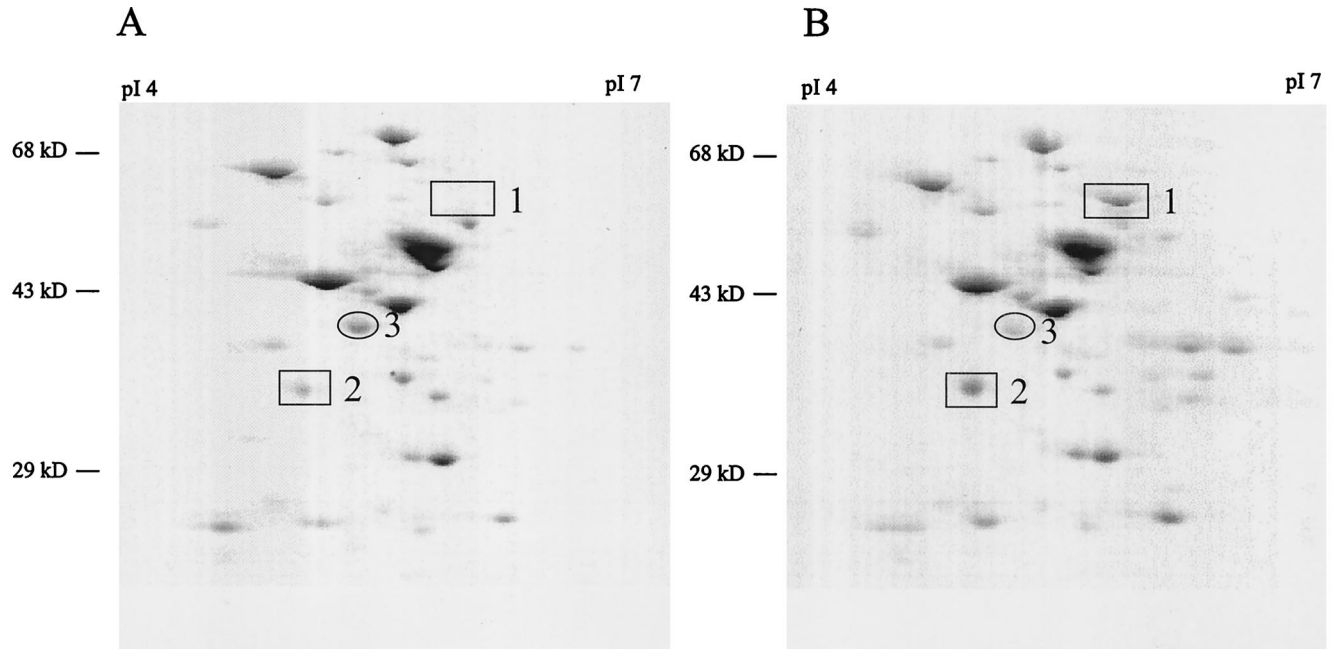

FIG. 1. Two-dimensional analysis of the cellular proteins of the opaque (A) and transparent (B) variants of pneumococcal clinical isolate p10. Bacterial proteins were separated by isoelectric focusing (pI 4 to 7) and gradient SDS-polyacrylamide gel electrophoresis (20 to 90 kDa). Circles and squares mark proteins with decreased and increased expression in the transparent variant, respectively, compared to the opaque variant. 1, 2, and 3 refer to the proteins discussed in the text.

RESULTS

Differentially expressed proteins in phenotypic variants of *S. pneumoniae*. The relative protein expression levels of phenotypic variants of strain p10 with opaque and transparent colony morphologies were compared using two-dimensional protein gel electrophoresis followed by computerized comparison of the CBB-stained gels. We performed this experiment with four cultures of opaque pneumococci and four cultures of transparent pneumococci. Approximately 200 distinct protein spots were analyzed. The quantity of two proteins, designated proteins 1 and 2, was decreased and the expression of protein 3 was increased in the opaque variant (Fig. 1). Protein 1 (65 kDa; pI, 5.4) was clearly detectable in the CBB-stained protein expression profile of the transparent variant but was not detectable in the opaque variant (Fig. 2). The quantity of protein 2 (35 kDa; pI, 5.0) in the transparent variant was 2.6 times the quantity of this protein in the opaque variant. In contrast, the quantity of protein 3 (40 kDa; pI, 5.1) was 2.1-fold greater in the opaque variant. The differences in the expression of proteins 1, 2, and 3 in the pneumococcal variants were statistically significant. The amounts of proteins 1, 2, and 3 were also compared in the phenotypic variants of clinical isolate strain p314 (serotype 6B). Again, proteins 1 and 2 were more prevalent in the transparent phenotype and protein 3 was more prevalent in the opaque phenotype; the levels of protein 1 and 2 were 1.3 times higher in the transparent variant and that of protein 3 was 1.4 times higher in the opaque variant. Although the relative expression levels of all three proteins were less pronounced in the p314 variants, the trend in the differential expression of the proteins is comparable between strains p10 and p314 (Fig. 2).

The expression of pyruvate oxidase is increased in transparent pneumococcal variants. Tryptic digestion products of protein 1 were analyzed by mass spectrometry. Nine amino acid sequences were identical to the amino acid sequence of the pneumococcal pyruvate oxidase, SpxB, except for leucine 389, which was reported as a serine by Spellerberg et al. (26) (GenBank accession number L39074) and as an asparagine by TIGR (Fig. 3). Since Spellerberg et al. analyzed the nucleotide sequence of *spxB* of strain R6x (26) and TIGR sequenced the genome of a type 4 pneumococcal strain, this discrepancy may be a strain-specific difference. The calculated SpxB molecular mass of 65,183 kDa correlated with the molecular ratio determined from two-dimensional protein electrophoresis, supporting the identity of the protein.

Since SpxB activity is known to result in the production of H_2O_2 (26), we measured H_2O_2 production in the phenotypic variants of strain p10. Opaque pneumococci produced <0.1 mmol of H_2O_2 per liter in 1 h, which is the detection limit of the assay, whereas transparent pneumococci produced 0.6 mmol of H_2O_2 per liter in the same period (5×10^4 CFU/ml). In controls, strain D39 generated 0.45 mmol of H_2O_2 per liter under similar conditions and the SpxB mutant of D39 produced <0.1 nmol of H_2O_2 per liter. The higher production of H_2O_2 by the transparent variant correlates with the difference in the amount of protein observed by comparison of protein expression patterns.

Increased expression of a proteinase maturation protein homologue in transparent pneumococcal variants. Mass spectrometric analysis of protein 2 resulted in seven peptides that were all identical to a putative translation product from the TIGR pneumococcal genome except for amino acid 142 (Fig. 4). This Tyr142-to-Gln/Lys substitution is most probably the result of strain-specific differences, since TIGR sequenced the genome of a type 4 pneumococcal strain. Since glutamine and lysine have the same residue mass, it is not possible to distinguish between them by mass spectrometry. The open reading frame encoding a hypothetical protein of 322 amino acids is located at nucleotides 7632 to 8597 on contig 33 of the TIGR sequence. The calculated molecular mass of this protein (35.4 kDa) is similar to the molecular ratio of protein 2 (35 kDa) identified by two-dimensional protein electrophoresis, sup-

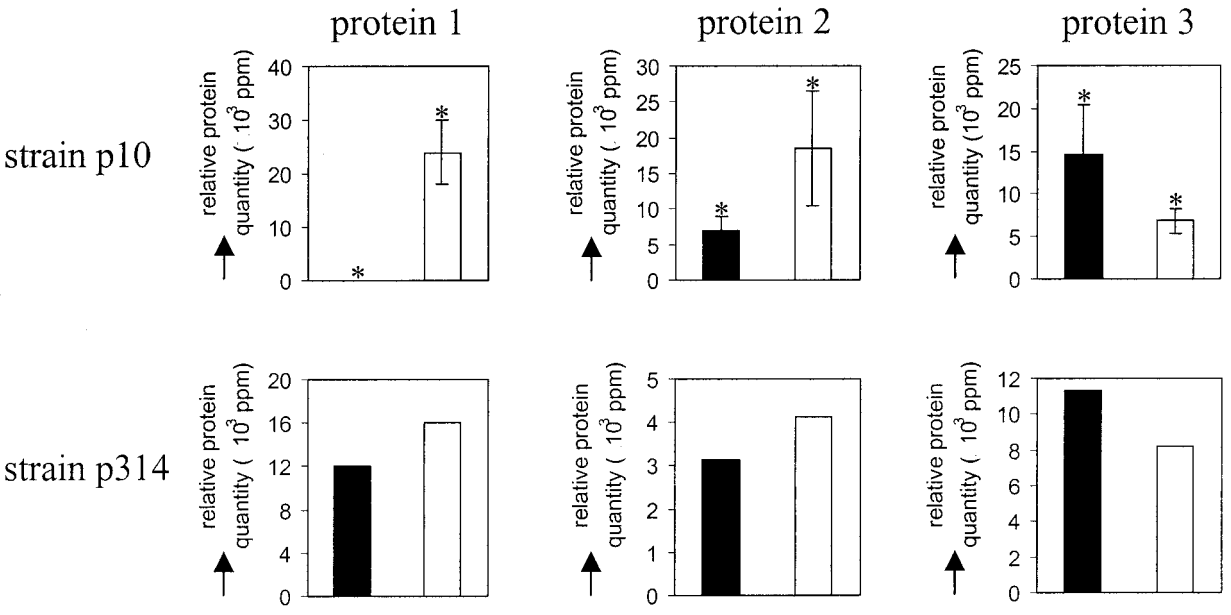

FIG. 2. Relative expression levels of proteins 1, 2, and 3 as repeatedly observed in four independent experiments with the opaque variant (solid bars) and the transparent variant (open bars) of pneumococcal clinical isolate p10 (serotype 9V) and observed in a single experiment with clinical isolate p314 (serotype 6B). Asterisks depict significant difference ($P \leq 0.05$) in the relative protein quantity in the opaque and transparent variants.

porting the identity of the protein. We designated this pneumococcal protein PpmA (for "putative proteinase maturation protein A") because it showed significant homology to the proteinase maturation protein (PrtM) of *Lactobacillus paracasei* (SWISS-PROT accession number Q02473) (Fig. 4). PrtM is essential for the production of active forms of the serine protease PrtP (8, 29). In *L. paracasei*, the gene encoding PrtP is located immediately downstream of *prtM* (11). In *S. pneumoniae*, no open reading frame homologous to serine proteases is found downstream of *ppmA*. PrtM is a membrane-bound lipoprotein in lactic acid bacteria (8). Also, *ppmA* contains a signal sequence of bacterial lipoproteins (Fig. 4).

Differences in the amount of PpmA in the phenotypic variants were also demonstrated by Western blot analysis. Whole-cell lysates from a *ppmA*-negative mutant and its parent strain demonstrated the PpmA band position (Fig. 5, lanes 1 and 2). When equivalent amounts of bacterial lysates of phenotypic variants of strains p10 and p314 were compared on Western blots, the amount of PpmA was larger in the transparent variants (lanes 4 and 6).

Reduced production of a putative elongation factor Ts in transparent pneumococcal variants. Mass spectrometric analysis of tryptic digestion products of protein 3 revealed three peptide sequences that were identical to a putative translation product from the TIGR pneumococcal genome (Fig. 6). The open reading frame encoding a hypothetical protein of 359 amino acids is located at nucleotides 911 to 1987 on contig 50 of *S. pneumoniae*. The calculated molecular mass (38.9 kDa) correlates with the molecular ratio of protein 3 (40 kDa) determined by two-dimensional protein electrophoresis. This protein showed significant homology to elongation factor Ts (EF-Ts) of *Helicobacter pylori* J99 (SWISS-PROT accession number AE001567) (Fig. 6).

001 MTQGKITASA AMLNVLKTWG VDTIYGIPSG TLSSLMDALA EDKDIRFLQV
051 RHEETGALAA VMQAKFGGSI GVAWGSGGPG ATHLINGVYD AAMDNTPFLA
101 ILGSRPVNEL NMDAFQELNQ NPMYNGIAVY NKRVAYAEQL PKVIDEACRA
151 AISKKGPAVV EIPVNFGFQE IDENSYYGSG SYERSFIAPA LNEVEIDKAV
201 E**ILNNAERPV** **IYAGF**GGVKA GEVITELSRK IKAPIITTGK NF**EAFEWNYE**
251 **GLTSAY**RVGW KPANEVVFEA DTVLFLGSNF AFAEVYEAFK NTEK**FIQVDI**
301 **DPYK**LGKRH**A** **LDASILGDAG** **QAA**KAILDKV NPVESTPWWR ANVKNNQNWR
351 DYMNKLEGKT EGE**LQLYQVY** **NAINK**HADQD **AIYSLDVGS**T TQTSTRHLHM
401 TPKNMWRTSP LF**ATMGIALP** GGIAAKKDTP DRQVWNIMGD GAFNMCYPDV
451 ITNVQYDLPV INLVFSNAEY GFIKNKYEDT NKH**LFGVDFT** NADYGKIAEA
501 QGAVGFTVDR IEDIDAVVAE AVKLNKGGKT VVIDARITQH RPLPVEVLEL
551 DPKLHSEEAI KAFKEKYEAE ELVPFRLF**LE** **EEGL**QSRAIK

FIG. 3. Amino acid sequence of the pneumococcal pyruvate oxidase (GenBank accession number L39074). The amino acid sequences of protein 1 derived by mass spectrometric analysis are depicted bold and underlined.

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Protein 2:  8   MKKKLLAGAITLLSVAT----LAACSKGSEGADLISMKGDVITEHQFYEQ
                MKKK+     + L S AT     L+ C          + +   G   +TE   FY++
PrtM      : 1   MKKKMRL-KVLLASTATALLLLSGCQSNQADQKVATYSGGKVTESNFYKE

Protein 2: 54   VKSNPSAQQVLLNMTIQKVFEKQYGSELDDKEVDDTIAEEKKQYGENYQR
                +K +P+ + +L NM I +      YG   +   K V+D      K+QYGEN+
PrtM      : 50   LKQSPTTKTMLANMLIYRALNHAYGKSVSTKTVNDAYDSYKQQYGENFDA

Protein 2: 104  VLSQAGMTLETRKAQIRTSKLVELAVKKVAEAELTDEAYKKAFDEYTPDV
                 LSQ G +   + K   +RT+ L E+A+KK+    ++++    K   +  Y P V
PrtM      : 100  FLSQNGFSRSSFKESLRTNFLSEVALKKL--KKVSESQLKAVWKTYQPKV

Protein 2: 154  TAQIIRLNNEDKAKEVLEKAKAEGADFAQLAKDNSTDEKTKENGGEITFD
                 T Q I L +++    + +     A G DFA LAK +S D   TK+NGG+I+F+
PrtM      : 148  TVQHI-LTSDEDTAKQVISDLAAGKDFATLAKTDSIDTATKDNGGKISFE

Protein 2: 204  SASTEVPEQVKKAAFALDVDGVSDVITATGTQAYSSQYYIVKLTKKTEKS
                 S +   +     K AA+ L      +                +              S
PrtM      : 197  SNNKTLDATFKDAAYKLKNGDYTQTPVKVTNGYEVIKMINHPAKGTFTSS

Protein 2: 254  SNIDDYKEKLKTVILTQKQNDSTFVQSIIGKELQAANIKVKDQAFQNIFT
                            K     +
PrtM      : 247  KKALTASVYAKWSRDSSIMQRVISQVLKNQHVTIKDKDLADALDSYKKPA

Protein 2: 304  QYI 306

PrtM      : 297  TTN 299
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FIG. 4. The hypothetical pneumococcal protein encoded by nucleotides 7632 to 8597 on contig 33 of the TIGR pneumococcal genome (protein 2) displays 41% sequence similarity to the proteinase maturation protein (PrtM) of *L. paracasei* (SWISS-PROT accession number Q02473). The putative lipoprotein signal sequence is underlined. The amino acid sequences of protein 2 derived by mass spectrometric analysis are depicted in bold.

DISCUSSION

Phenotypic variation is an important mechanism that allows bacterial pathogens to adapt to different host environments. The ability to survive in different host environments is the result of molecular adaptation, e.g., changes in the expression of specific cell surface components (10, 31). The expression of four previously identified proteins has been examined in phase variants of *S. pneumoniae*. PspA is expressed in larger amounts in the opaque phenotype, whereas CbpA and LytA are expressed in larger amounts in the transparent phenotype (13, 21, 33). No difference in pneumolysin expression has been found by Western blot analysis of whole-cell lysates of the opaque and transparent variants (13). In this study, we identified and characterized three additional proteins that are differentially expressed in phenotypic variants of *S. pneumoniae*. The minimal protein amount necessary for detection and identification by mass spectrometric analysis is about 500 fmol. For this reason, gels were stained with CBB and not by the more sensitive silver-stain method, since only proteins visualized by CBB staining reached the mass spectrometry analysis detection level. This may explain why we did not confirm the differential expression of LytA, PspA, and CbpA in the phenotypic variants and why we identified differential expression of three proteins only.

We demonstrated that the expression of pyruvate oxidase was increased in the transparent variants. Pyruvate oxidases are crucial for aerobic carbohydrate metabolism of several streptococci (4). This enzyme decarboxylates pyruvate to acetyl phosphate, resulting in the release of H_2O_2 and CO_2. In *S. pneumoniae*, the release of H_2O_2 is mainly the result of SpxB activity, since a *spxB*-defective mutant produces virtually no H_2O_2 in comparison with the parent strain (26). Therefore, the higher expression of SpxB in the transparent phenotype most probably explains the increased production of hydrogen peroxide. Spellerberg et al. have identified pyruvate oxidase as an indirect determinant of virulence in *S. pneumoniae* (26). A *spxB* mutant has a decreased ability to colonize the nasopharynx in a rabbit model. This is reflected by the lower capacity of binding to the nasopharyngeal cells and to their glycoconjugate receptors. The decreased ability of *spxB*-defective pneumococcal mutants to colonize the nasopharynx and the increased expression of SpxB in transparent variants suggests the potentially important role of SpxB in efficient colonization of the nasopharynx by transparent variants (26). The expression level of SpxB is unlikely to directly determine colony morphology, since the *spxB* mutant of D39 still varies in colony morphology (J. N. Weiser, unpublished data).

The second protein with increased expression in the transparent variant demonstrated significant sequence homology to

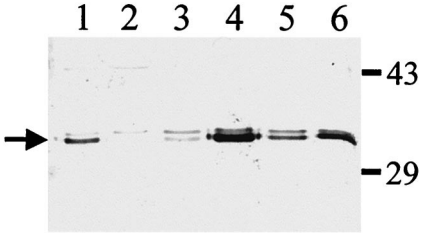

FIG. 5. Western blot analysis of phenotypic variants with anti-PpmA rabbit serum. The amount of PpmA was compared in equivalent amounts of whole-cell lysates of opaque (lanes 3 and 5) and transparent (lanes 4 and 6) variants of pneumococcal clinical isolate p10 (lanes 3 and 4) and p314 (lanes 5 and 6). The *ppmA*-deficient mutant (lane 2) and its parent strain D39 (lane 1) were used as controls. Numbers indicate the molecular size markers (in kilodaltons). The arrow indicates the PpmA protein band.

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Protein 3:  14  MAEITAKLVKELREKSGAGVMDAKKALVETDGDIEKAIELLREKGMAKAA
                M+ I+A+LVK+LR+ + AG+MD KKALVE  GD++KAI+ LREKG++KAA
EF-Ts     : 1   MSGISAQLVKKLRDLTDAGMMDCKKALVEVAGDLQKAIDFLREKGLSKAA

Protein 3:  64  KKADRVAAEGLTGVYVNGNV--AAVIEVNAETDFVAKNAQFVELVNTTAK
                KKADR+AAEG+  + V  +   A ++E+N+ETDFVAKN  F ELV  T +
EF-Ts     : 51  KKADRIAAEGVVALEVAPDFKSAMMVEINSETDFVAKNEGFKELVKKTLE

Protein 3:  112  VIAEGKPANNEEALALIMPSGETLEAAYVSATATIGEKISFRRFALIEKT
                 I        EE L   + + +  E    S  A IGE I  R+ A ++
EF-Ts     : 101 TIKTHNIHTTEELLKSPLDN-KPFEEYLHSQIAVIGENILVRKIAHLKAP

Protein 3:  162 DAQHFGAYQHNGGRIGVISVVEGGDEA-------LAKQLSMHIAAMKPTV
                      +Y H+  R+GV+  +E  +E         LA+ ++MH AAMKP V
EF-Ts     : 151 SSHIINGYAHSNARVGVLIAIEYNNEKNAPKVVELARNIAMHAAAMKPQV

Protein 3:  205 LSYKELDEQFVKDELAQLNHVIDQDNESRAMVNKPALPHLKYGSKAQLTD
                L  K+    FVK E   L   I++DNE    + KP       +GS+ +L+D
EF-Ts     : 201 LDCKDFSLDFVKKETLALIAEIEKDNEEAKRLGKPLKNIPTFGSRIELSD

Protein 3:  255 DVIAQAEADIKAELAAEGKPEKIWDKIIPGKMDRFMLDNTKVDQAYTLLA
                +V+A  +   + EL  +GKPEKIWDKI+PGKM+RF+ DNT +DQ  TLL
EF-Ts     : 251 EVLAHQKKAFEDELKEQGKPEKIWDKIVPGKMERFIADNTLIDQRLTLLG

Protein 3:  305 QVYIMDDSKTVEAYLESV------NASVVEFARFEVGEGIEKAANDF
                Q Y+MDD KT+   +         N  + E+ RFE+GEGIEK   +F
EF-Ts     : 301 QFYVMDDKKTIAQVIADCSKEWDDNLKITEYVRFELGEGIEKKTENF
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FIG. 6. The hypothetical pneumococcal protein encoded by nucleotides 911 to 1987 on contig 50 of the TIGR pneumococcal genome (protein 3) displays 61% sequence similarity to EF-Ts of *H. pylori* J99 (SWISS-PROT accession number AE001567). The amino acid sequences of protein 3 derived by mass spectrometric analysis are depicted in bold.

proteinase maturation protein (PrtM) of *L. paracasei. L. paracasei* and other lactic acid bacteria are used in the food industry for the production of a variety of fermented milk products. During growth, these bacteria produce cell envelope-located serine proteases that break down caseins, the major proteins in milk. PrtM is a *trans*-acting protein involved in the maturation (processing into active proteins) of serine protease, PrtP (29). PrtM belongs to the family of peptidyl-prolyl *cis*/*trans* isomerases that are thought to assist in protein folding by catalyzing the *cis-trans* isomerization of the peptidyl-prolyl bonds in peptides and proteins (22). PpmA contains an N-terminal lipoprotein signal sequence, which suggests that PpmA, like PrtM (8), is membrane bound. We hypothesize that PpmA also functions as a membrane-bound molecular chaperone. In *L. paracasei*, the gene encoding PrtP is located immediately downstream of *prtM* (11). Both *prtM* and *prtP* were found to be transcribed from the same promoter region but in opposite directions (29). In *S. pneumoniae*, no open reading frame homologous to a serine protease could be located in the direct vicinity of *ppmA*. The pneumococcal proteins activated by PpmA are currently unknown. We demonstrated differential expression of PpmA in the phenotypic variants of *S. pneumoniae*, which suggests that PpmA may play a role in the pathogenesis of infections. PpmA is more prevalent in the transparent phenotype that is selected for during nasopharyngeal colonization, suggesting that PpmA is directly involved in adherence through maturation of surface proteins with adherence properties or indirectly by the activation of proteases or other secreted proteins.

The expression of the third protein, identified as EF-Ts, was increased in the opaque phenotype. EF-Ts is essential for the elongation of the polypeptide chain during protein synthesis. The protein mediates the regeneration of EF-Tu · GDP into the active form, EF-Tu · GTP. This active form of EF-Tu facilitates the entry of aminoacyl-tRNA to the ribosome, enabling protein synthesis. Differential expression of EF-Ts has been found previously in the gram-negative bacterium *Coxiella burnetii* (25), the causative agent of Q fever. This obligate intracellular parasite replicates in distinct morphological forms that may allow potential life cycle variants to survive the harsh environment of the phagolysosome. Two distinct morphological forms of *C. burnetii* have been described, a large-cell variant and a small-cell variant. Large-cell variants are metabolically more active than small-cell variants (15). This is supported by data showing that EF-Ts and EF-Tu were more prevalent in large-cell variants (25). We hypothesize that the increased presence of EF-Ts in the opaque variants of *S. pneumoniae* indicates that, like in *C. burnetii*, the opaque variants are metabolically more active, which may explain the rapid invasive growth characteristics of these variants.

This study shows that the combination of two-dimensional protein gel electrophoresis, mass spectrometry, and genomics is a powerful tool for the identification of differentially expressed proteins in phenotypic variants of *S. pneumoniae*. We have identified differential expression of pyruvate oxidase, a new member of the family of peptidyl-prolyl *cis*/*trans* isomerases (PpmA), and EF-Ts in pneumococcal phenotypic variants of type 9V and 6B clinical isolates. We hypothesize that the higher expression of both pyruvate oxidase and PpmA in the transparent phenotype correlates with increased adhesive properties and ability to colonize the nasopharynx. The higher expression of EF-Ts in the opaque variant might indicate a higher metabolic activity.

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