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Identification of Two Proteins Associated with Virulence of *Streptococcus suis* Type 2

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The protein profiles of various cell fractions of 180 strains of *Streptococcus suis* type 2, which were isolated from diseased pigs, from healthy pigs when they were slaughtered, and from human patients, were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting. The isolates from diseased pigs contained two proteins that were absent in most of the isolates from healthy pigs. One of these proteins was a 136-kDa protein that was previously identified as the muramidase-released protein (MRP). This protein was predominantly detected in protoplast supernatants and culture supernatants. The second protein was a 110-kDa protein that was detected only in culture supernatants and therefore was provisionally called extracellular factor (EF). Three phenotypes of *S. suis* type 2 strains were recognized. Isolates from organs of diseased pigs mainly belonged to the MRP$^+$ EF$^+$ phenotype (77%), while isolates from tonsils of healthy pigs mainly had the MRP$^-$ EF$^-$ phenotype (86%). Most of the isolates from human patients contained MRP (89%); 74% had the MRP$^+$ EF$^-$ phenotype. These findings confirm the results of previous investigations which demonstrated that *S. suis* type 2 strains differ in virulence. Monoclonal antibodies raised against the 110-kDa EF recognized proteins with higher molecular weights in culture supernatants of all of the strains with the MRP$^+$ EF$^-$ phenotype. However, none of the strains with the MRP$^+$ EF$^+$ phenotype produced these high-molecular-weight proteins. Our results demonstrate that MRP and EF are associated with virulence. This suggests that one or both of these proteins are virulence factors that play a role in the pathogenesis of *S. suis* type 2 infections in pigs and human patients.

Streptococcus suis type 2 infection causes various pathological and clinical signs of disease in pigs (4, 21, 24, 25) and humans (3, 15, 26). *S. suis* type 2 infection can cause septicemia, meningitis, arthritis, and sudden death in young pigs after weaning, but the infection can also be limited to the respiratory tract, where it causes bronchopneumonia (16, 21). *S. suis* type 2 infection in humans causes septicemia and meningitis, which is often complicated by arthritis, endophthalmitis, and cochleitis (3, 26). Adult pigs can harbor the streptococci in the palatine tonsils with no signs of disease (5). Although the proportion of carrier pigs in a herd may be high, this proportion does not correlate with a high incidence of disease (6). Arends et al. reported that 32 to 50% of healthy pigs when they were slaughtered were carriers of *S. suis* type 2 (2). The authors of most studies, including studies of herds with endemic *S. suis* type 2 infections, have not reported such high rates of infection, however (5, 6). The variation in these rates of infection may have been caused by differences in the virulence of various strains of *S. suis* type 2. In a previous study we demonstrated that the virulence properties of two *S. suis* strains differed distinctly in newborn germfree pigs (20). Because the cell wall of the virulent strain contained a high-molecular-weight, muramidase-released protein (MRP) that was absent in the nonvirulent strain, we suspected that this MRP is important in the pathogenesis of *S. suis* type 2 infections in pigs.

In this study we compared the protein profiles of 180 strains of *S. suis* type 2 that were isolated from pigs and human patients. We carried out this study in order to evaluate the evidence for the role of MRP as a virulence factor and to identify other proteins that may be virulence factors.

MATERIALS AND METHODS

Streptococcal isolates. The 180 strains of *S. suis* type 2 were obtained from three different sources. A total of 111 of these strains were obtained from four Animal Health Services in The Netherlands. These strains were isolated from organs of diseased pigs in the course of routine diagnostic procedures. Another 42 strains were isolated from the tonsils of healthy pigs when they were slaughtered. In addition, 27 strains were isolated from human patients with *S. suis* type 2 infections. Tonsillar and human strains were kindly provided by J. P. Arends, Streeklaboratorium voor de Volksgezondheid voor Groningen en Drente, Groningen, The Netherlands. All strains were typed as *S. suis* type 2 by using biochemical and serological methods, as described previously (21). Strain 1 (=D-282) had been determined previously to be virulent for newborn germfree pigs and produced MRP, whereas strain 2 (=T-15) was nonvirulent and did not produce MRP (20). Therefore, strains 1 (MRP$^+$) and 2 (MRP$^-$) were used as reference strains.

Culture conditions. A 1-day-old colony of each bacterial strain was grown on Columbia blood agar base (code CM 331; Oxoid, Ltd., Inc., Columbia, Md.) containing 6% horse blood and was incubated overnight at 37°C in Todd-Hewitt broth (code CM 189; Oxoid). Early-stationary-growth-phase cultures were obtained from the overnight cultures, diluted 10 times in Todd-Hewitt broth, and incubated for 4 h at 37°C.

Cell fractionation. Two cell fractions (protoplast supernatant and culture supernatant) were prepared from each of the

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180 strains. Two additional cell fractions (protoplasts and membrane vesicles) were prepared from 23 strains that were selected randomly from the 180 strains. The 23 strains were isolated from both diseased and healthy pigs, as well as from human patients.

The four cell fractions were isolated from early-stationary-growth-phase cultures in Todd-Hewitt broth. Protoplasts were isolated as described by van der Vossen et al. (18). After centrifugation in an Eppendorf centrifuge, the protoplasts and the remaining supernatants (protoplast supernatant) were collected. Membrane vesicles were isolated essentially as described by Driessen et al. (7). The broth cultures were centrifuged at 4,000 × *g* for 15 min, and the culture supernatants were collected.

Preparation of antigens and antisera. After a stationary-growth-phase culture of strain D-282 was centrifuged, the supernatant was harvested, concentrated by filtration (type PM30 filters; Amicon Corp., Danvers, Mass.) to a concentration of 3 mg/ml, and dialyzed once against Tris-buffered saline (50 mM, pH 7.5). This product was used as an antigen for raising polyclonal antibodies (PAb) in rabbits and monoclonal antibodies (MAb) in mice. Rabbits were immunized by intramuscular and subcutaneous inoculation of 2-mg portions of protein emulsified in equal volumes of Freund incomplete adjuvant. Inoculations were repeated the following day without the adjuvant. After 5 weeks the rabbits were given intravenous booster inoculations of the same antigen dose, but without the adjuvant. After 6 weeks, the rabbits were exsanguinated. The serum of one rabbit (rabbit K191) was used as a probe in the Western blot analysis.

MAb against a protein designated extracellular factor (EF) (see below) were raised in BALB/c mice. The mice were immunized intraperitoneally with 0.5-ml portions of antigen containing 25 μg of protein emulsified in equal volumes of Freund incomplete adjuvant; 3 weeks later this procedure was repeated. After 5 weeks, the mice were given intravenous booster inoculations of the same antigen dose, but without the adjuvant. Hybridoma cell lines were prepared as described by van Zijderveld et al. (19). After being grown for 10 to 14 days, hybridomas were tested for antibodies against EF by using an enzyme-linked immunosorbent assay. Hybridoma culture supernatants (diluted 1:2) were then tested for anti-EF MAb on Western blots of culture supernatants from strain D-282. Binding of MAb to the 110-kDa protein on the nitrocellulose filters was visualized with anti-mouse immunoglobulins conjugated with alkaline phosphatase as described below. The positive cells were cloned twice by limiting dilution in microtiter plates. The resulting monoclonal cell lines were used to produce ascites fluid in pristane-primed male BALB/c mice, as described previously (19).

Indirect enzyme-linked immunosorbent assay for screening hybridoma culture supernatants. Polystyrene microtiter plates (Greiner, Nürtingen, Germany) were coated for 16 h at 37°C with a solution containing the concentrated, dialyzed culture supernatant from strain D-282 (see above) diluted in phosphate-buffered saline (pH 7.2; 0.075 mg of protein per ml), and then these preparations were incubated for 16 h at 37°C. Twofold dilutions of hybridoma culture supernatants were applied and tested as described previously (19). Bound antibodies were incubated with anti-mouse immunoglobulins (diluted 1:500) that were conjugated with horseradish peroxidase (Nordic, Tilburg, The Netherlands).

Electrophoresis and Western blotting. The various cell fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described by Laemmli (11); the separating gels contained 6 or 12% polyacrylamide. After electrophoresis, the proteins were stained with silver (13). For Western blot analysis, the proteins were electroblotted onto nitrocellulose by using a Multiphor II Nova Blot system according to the recommendations of the manufacturer (Pharmacia LKB, Uppsala, Sweden). The blots were probed with a 1:500 dilution of rabbit K191 PAb or with a 1:300 dilution of mouse MAb. Bound PAb were visualized with anti-rabbit immunoglobulins conjugated with alkaline phosphatase. Bound MAb were visualized with a 1:1,000 dilution of anti-mouse immunoglobulins conjugated with alkaline phosphatase (Zymed Laboratories, Inc., San Francisco, Calif.).

RESULTS

Protein profiles of four cell fractions of 23 selected strains. Figure 1A shows the protein profiles of the protoplast supernatants, culture supernatants, and membrane vesicles from two *S. suis* isolates belonging to each group studied (diseased pigs, healthy pigs, and human patients). The protein profiles of the protoplast (data not shown) and membrane vesicle cell fractions prepared from the 23 strains which we examined were almost identical. In contrast, the protein profiles of the culture and protoplast supernatants differed distinctly. The protein profiles of isolates obtained from diseased pigs contained two protein bands that were absent in the protein profiles of most isolates obtained from healthy pigs (Fig. 1A, arrows). One band represented a 136-kDa protein, which we identified previously as MRP (20). In the sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis, separating gels containing 6% polyacrylamide revealed the presence of MRP in both culture and protoplast supernatants (Fig. 1A, strains 1, 5, 24, and 26). The second band represented a 110-kDa protein; because this protein was detected only in culture supernatants, it was designated EF. Both MRP and EF were present in the culture supernatant of virulent reference strain 1 (=D-282) (Fig. 1A, lane 1), but were absent in all cell fractions of nonvirulent reference strain 2 (=T-15) (Fig. 1A, lane 2). The eight strains isolated from diseased pigs contained both MRP and EF. Six of the eight strains isolated from healthy pigs lacked these proteins. Six of the seven strains isolated from human patients contained MRP, but only three of the six also contained EF.

When rabbit K191 PAb directed against culture supernatants were used as probes in the immunoblotting analysis, MRP and EF were clearly detected in the cell fractions of *S. suis* type 2 strains. Protoplast supernatants, culture supernatants, and membrane vesicles of strains 1, 5, 24, and 26 contained the 136-kDa MRP (Fig. 1B). Because the 136-kDa MRP is a major component of protoplast supernatants, this protein must be localized in the cell envelope of the bacteria. The culture supernatants of strains 1 and 5 also contained the 110-kDa EF. Strains 24 and 26 contained MRP but not EF; strains 2 and 13 contained neither of the proteins (Fig. 1B).

On the basis of the presence of MRP and EF in culture supernatants, the following three phenotypes of *S. suis* type 2 strains were distinguished: MRP^+ EF^+, MRP^+ EF^-, and MRP^- EF^- (Fig. 2A). Proteins bands at various molecular masses higher than 150 kDa reacted with rabbit K191 serum and were visualized in Western blots of culture supernatants of strains 17, 24, 25, 26, and 28. As such proteins were also recognized by our anti-EF MAb, except in the culture supernatant of strain 25, the 110-kDa EF was probably

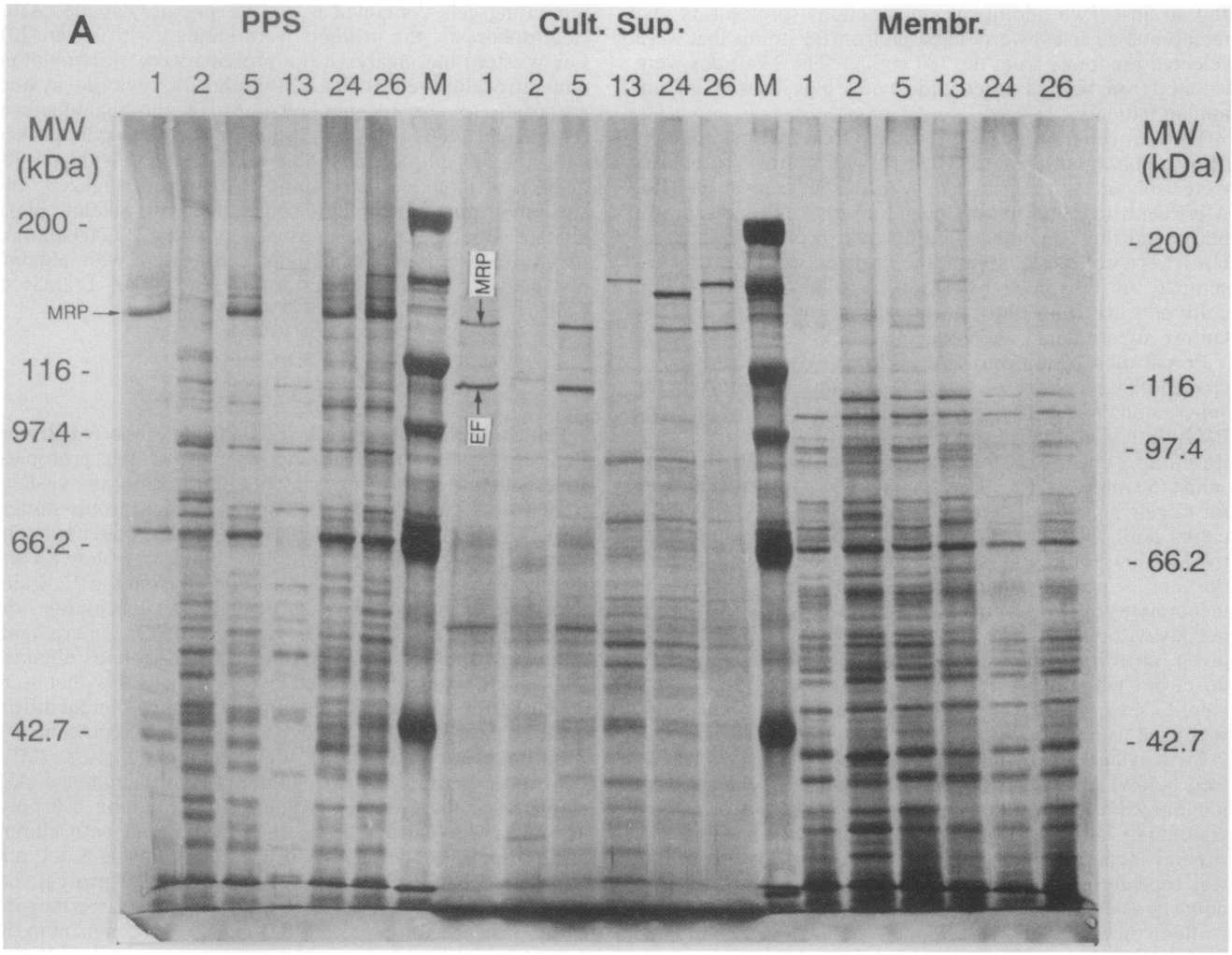

FIG. 1. (A) Protein profiles of protoplast supernatants (PPS), culture supernatants (Cult. Sup.), and membrane vesicles (Membr.) of *S. suis* type 2 strains analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 6% polyacrylamide slab gel stained with silver. The positions of MRP (136 kDa) and EF (110 kDa) are indicated by arrows. The lane designations are strain designations except for lane M. Reference strain 1 (=D-282) and strain 5 were isolated from pigs with meningitis. Reference strain 2 (=T-15) and strain 13 were isolated from the tonsils of healthy pigs. Strains 24 and 26 were isolated from human patients. We used molecular mass markers (42.7 to 200 kDa) from Bio-Rad Laboratories, Richmond, Calif. (B) Western blot of the protoplast supernatant (PPS), culture supernatant (Cult. Sup.), and membrane vesicle (Membr.) fractions probed with anti-MRP/EF rabbit K191 serum (diluted 1:500). The lane designations are strain designations.

related to these proteins (Fig. 2B). Western blots probed with the mouse anti-EF MAb showed that all of the strains with the MRP^+ EF^- phenotype contained higher-molecular-weight proteins in their culture supernatants. However, none of the strains with the MRP^+ EF^+ phenotype contained such proteins. After probing with rabbit K191 serum, we detected certain high-molecular-weight proteins in culture supernatants of 12 MRP^- EF^- strains, including strain 25. Immunoblotting with anti-EF MAb showed that these proteins were not related to EF.

When the four cell fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12% slab gels, no low-molecular-weight proteins associated with virulence were detected (data not shown).

Protein profiles of culture and protoplast supernatants of 180 strains. All 180 *S. suis* type 2 strains were analyzed for the occurrence of the three phenotypes in culture and protoplast supernatants by using 6% slab gels. Eighty percent of the strains isolated from the organs of diseased pigs had the MRP^+ EF^+ phenotype (Table 1). In contrast, only 2% of the strains isolated from tonsils of healthy pigs had this phenotype; 86% of these strains were MRP^- EF^-. Only 15% of the strains isolated from human patients had the MRP^+ EF^+ phenotype. Among the *S. suis* type 2 strains tested, far more human strains (74%) than porcine strains (12%) had the MRP^+ EF^- phenotype; 89% of the human strains were MRP^+. The MRP^- EF^+ phenotype was not detected.

DISCUSSION

In this study, we analyzed the protein profiles of four cell fractions of 23 *S. suis* type 2 strains isolated from three groups: diseased pigs, healthy pigs, and human patients.

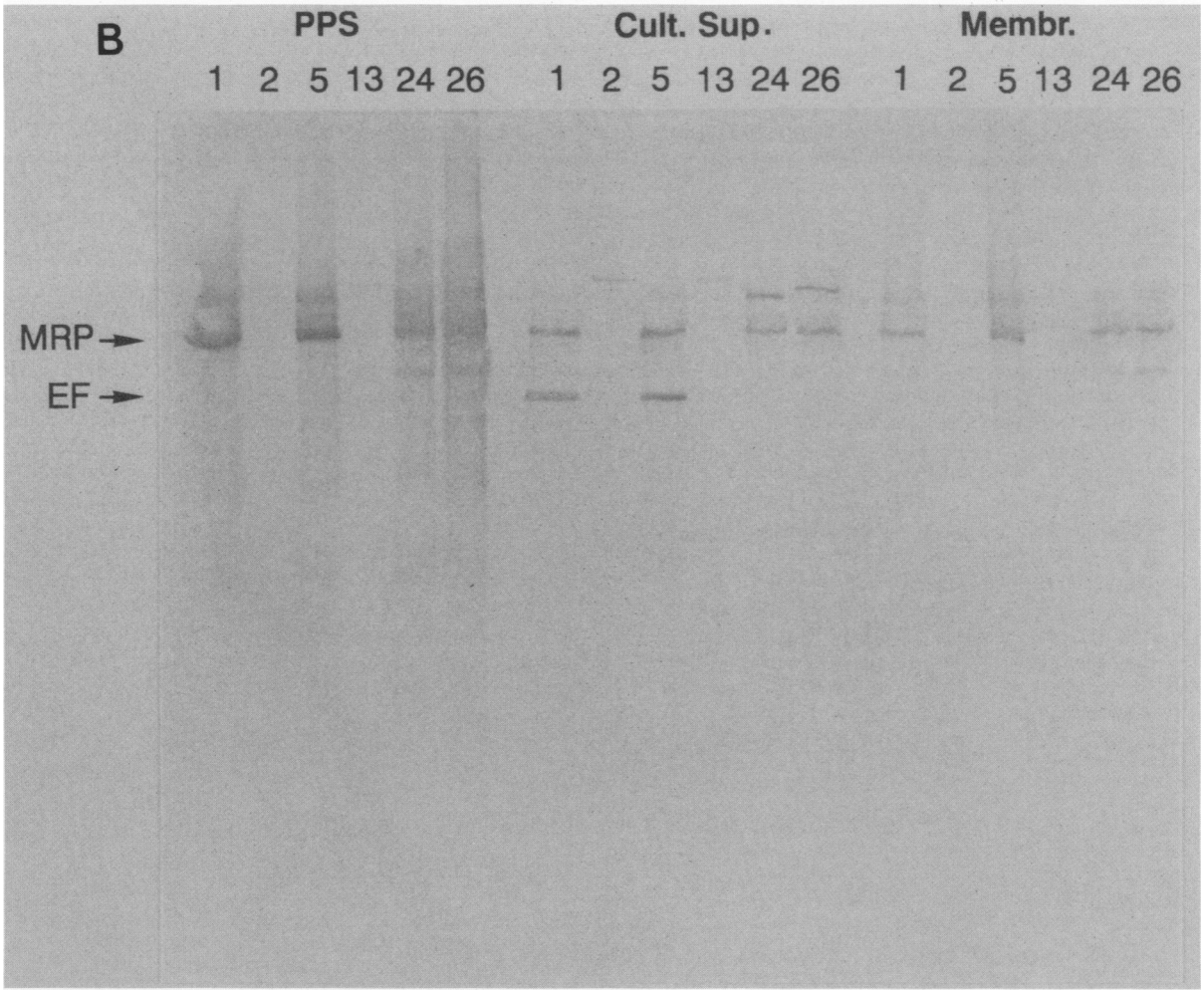

FIG. 1—*Continued.*

These strains included virulent reference strain 1 (=D-282) and nonvirulent strain 2 (=T-15) (20). By comparing protein profiles within as well as between groups, we found that EF and MRP were predominantly present in culture or protoplast supernatants of strains isolated from diseased pigs. Therefore, we considered these two proteins to be potential virulence factors. Because culture and protoplast supernatants showed the presence of MRP and EF most clearly, we chose these fractions to examine additional strains. The presence or absence of MRP and EF was used as a basis for distinguishing three phenotypes in *S. suis* type 2 strains. The occurrence of these phenotypes in 180 strains was studied (Table 1). Most of the strains isolated from diseased pigs contained both MRP and EF, which suggests that these proteins are virulence factors. However, we cannot exclude the possibility that these proteins merely occur in association with true virulence factors. Most strains isolated from diseased pigs and from human patients contained MRP, but only 15% of the strains isolated from human patients contained EF. Recent experiments have demonstrated that MRP^+ EF^+ strains are more pathogenic for pigs than MRP^+ EF^- strains are (22). This finding suggests that MRP and EF are not equally important in the pathogenesis of *S. suis* type 2 infections, either in pigs or in humans; EF may be more specific for strains that affect pigs than for strains that affect humans. Because 11% of the strains isolated from diseased pigs and from human patients did not contain MRP or EF, factors other than these proteins may also be important in the pathogenesis of *S. suis* type 2 infections.

Arends and Zanen described lysozyme-positive proteins in isolates from humans and pigs (1). It is highly likely that MRP and lysozyme-positive proteins are the same, since the tonsillar and human strains used in this study were obtained from J. Arends. Since MRP appears predominantly in protoplast supernatants, it is probably a protein that is associated with peptidoglycan. It was probably this antigen, among others, that was recognized by sera of pigs immune to *S. suis* type 2 infection (10). The molecular weight of the MRP in the culture supernatant fraction was somewhat lower than the molecular weight of the MRP in the protoplast supernatant fraction. Being a cell wall protein, MRP may break from the peptidoglycan layer during the growth of the bacteria, resulting in unbound MRP in the culture supernatant that has a lower molecular weight than bound MRP in the protoplast supernatant (Fig. 1A). Peptidoglycan-associated proteins of many streptococci have been described previously (for example, the M protein of group A streptococci) (14). The M protein is an important virulence factor, because only

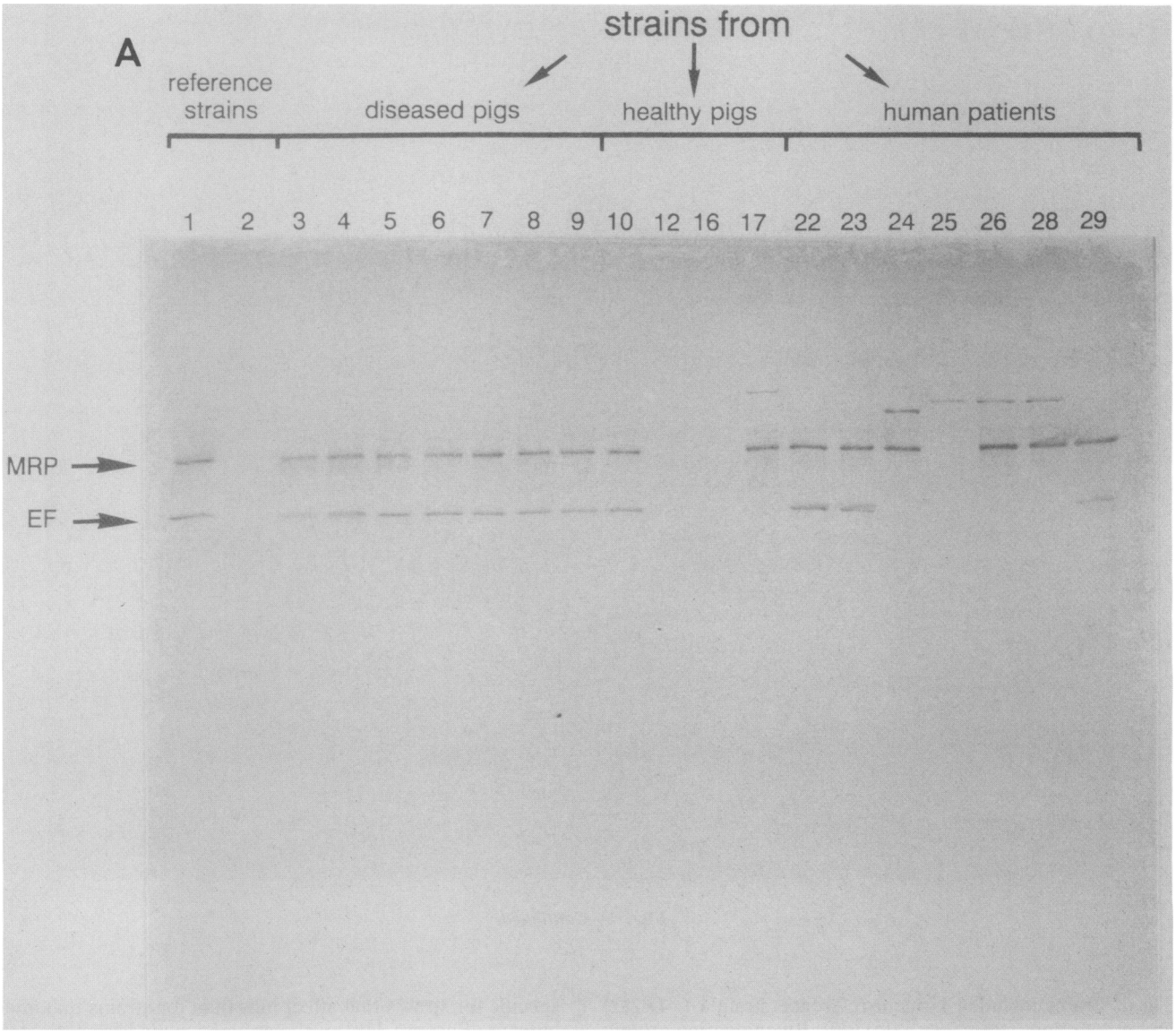

FIG. 2. (A) Western blot of the cell culture supernatants of selected *S. suis* type 2 strains probed with rabbit anti-MRP/EF serum (K191), anti-MRP serum, and anti-EF serum (1:500 diluted). The PAbs revealed the following three *S. suis* type 2 phenotypes: MRP^+ EF^+, MRP^+ EF^-, and MRP^- EF^-. The lane numbers are strain designations. Reference strain 1 (=D-282) and strains 3 to 9 (MRP^+ EF^+) were isolated from pigs with *S. suis* meningitis. Reference strain 2 (=T-15) and strains 10, 12, 16, and 17 were isolated from the tonsils of healthy pigs. Strains 22, 23, 24, 25, 26, 28, and 29 were isolated from human patients. (B) Western blot of the cell culture supernatants of selected *S. suis* type 2 strains probed with mouse anti-EF MAb (diluted 1:300). The lane numbers are strain designations. The MAb recognized both the 110-kDa EF and the higher-molecular-mass proteins of strains 17, 24, 26, and 28. These higher-molecular-mass proteins (>150 kDa) were identical to those shown in panel A.

M-positive strains resist phagocytosis and adhere to human buccal epithelial cells (8, 12). Williams (23) reported that only virulent strains of *S. suis* type 2 survived within mouse macrophages. The M protein varies in its antigenic composition and size (9). MRP might vary similarly.

Because EF appears only in culture supernatants, it is obviously an extracellular, secreted protein. Since our unabsorbed rabbit serum showed the presence or absence of MRP and EF so clearly, these proteins are probably the main antigenic components in the culture supernatants of virulent *S. suis* type 2 strains. Several other high-molecular-mass proteins (>150 kDa) were also detected in the culture supernatants of tonsillar strain 17 and human strains 24, 25, 26, and 28 probed with rabbit K191 serum (Fig. 2A). Western blots of these fractions probed with the mouse anti-EF MAb showed that these proteins share epitopes with EF (Fig. 2B). In addition, DNA probes containing the gene that encodes EF hybridize with the genes that encode both these higher-molecular-weight proteins and the 110-kDa EF. These preliminary data indicate that these proteins are related to EF (17). This implies that at least a part of the EF gene of strains with an MRP^+ EF^- phenotype is homologous with the EF gene of strains with an MRP^+ EF^+ phenotype. Apparently the length of the EF gene varies in the different *S. suis*

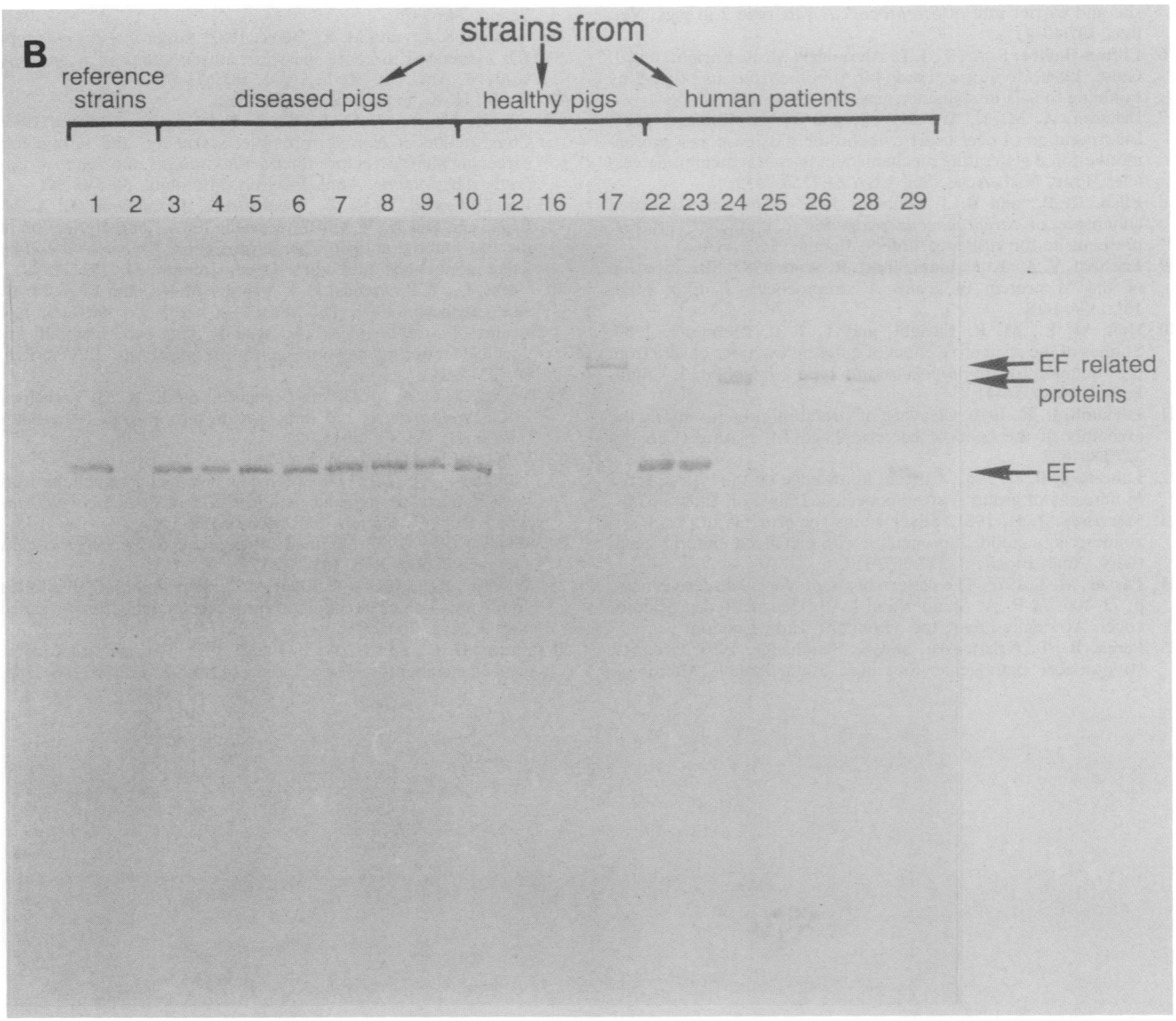

FIG. 2—*Continued.*

strains. All of the strains with an MRP$^+$ EF$^-$ phenotype contained the higher-molecular-mass form of EF, but none of the strains with an MRP$^+$ EF$^+$ phenotype did. These findings indicate that only the lower-molecular-mass form of EF (110 kDa) is active in the pathogenesis of *S. suis* infection in pigs.

TABLE 1. Prevalence of MRP and EF phenotypes in 180 streptococcal strains isolated from diseased pigs, from healthy pigs when they were slaughtered, and from human patients

S. suis type 2 phenotype	No. (%) of strains isolated from:		
	Organs of diseased pigs	Tonsils of healthy pigs	Human patients
MRP$^+$ EF$^+$	86 (77)	1 (2)	4 (15)
MRP$^+$ EF$^-$	13 (12)	5 (12)	20 (74)
MRP$^-$ EF$^-$	12 (11)	36 (86)	3 (11)

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