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Mutants of *Streptococcus suis* Types 1 and 2 Impaired in Expression of Muramidase-Released Protein and Extracellular Protein Induce Disease in Newborn Germfree Pigs

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The contribution of muramidase-released protein (MRP) and extracellular factor (EF) to the virulence of *Streptococcus suis* type 1 and 2 infections was studied. For that aim, we constructed mutants of *S. suis* types 1 and 2 by inactivating the genes encoding MRP and EF. Moreover, we changed a type 2 strain producing the 110-kDa EF protein into a strain producing a modified protein (EF*) of increased molecular mass. The chromosomally located *mrp* and *epf* genes were inactivated by replacement recombination by using nonreplicative plasmids. Newborn germfree pigs were inoculated with pathogenic type 1 and 2 strains and with the isogenic mutant strains. Wild-type as well as mutant strains induced fever, specific signs of disease, and lesions. Moreover, all mutant strains could be reisolated from the central nervous system of infected pigs. These results showed that inactivation or alteration of the *mrp* and *epf* genes had no measurable effect on the pathogenicity of *S. suis* types 1 and 2.

Streptococcus suis is an important cause of meningitis, septicemia, arthritis, and sudden death in young pigs (3, 21) and of meningitis in humans (1). Little is known about the pathogenesis and the epidemiology of the disease. So far, no suitable diagnostic tests or vaccines are available for the control of this infectious disease.

To date, 32 capsular serotypes of *S. suis* have been described (5, 7). Within the various serotypes, the virulence of strains may differ (20, 25). We previously identified two proteins of *S. suis* serotype 2 that seemed to be associated with pathogenicity (23). On the basis of the presence or absence of these proteins, three phenotypically different strains were distinguished (23, 25). First, strains were found that produce a 136-kDa cell envelope-associated protein, designated muramidase-released protein (MRP), and a 110-kDa extracellular protein factor (EF). Strains of this phenotype (MRP^+ EF^+) were frequently isolated from organs of diseased pigs. They induced specific clinical signs of disease in newborn germfree pigs after experimental infection. Second, strains were found that produce neither MRP nor EF. Strains of this phenotype (MRP^- EF^-) were frequently isolated from tonsils of healthy pigs and did not induce any signs of disease in piglets after experimental infection. Strains of the third phenotype produce MRP but not the 110-kDa EF protein. Instead, these strains produce EF-related (EF*) proteins of various sizes. These strains, with the phenotype MRP^+ EF*, were frequently isolated from human patients. The MRP^+ EF* strains, however, did not induce severe clinical signs of disease in piglets after experimental infection.

Strains of serotype 1 had either the MRP^s EF^+ or the MRP^- EF^- phenotype. MRP^s EF^+ strains produced an MRP with a reduced size (molecular mass, about 120 kDa) next to the 110-kDa EF protein. Those strains were highly pathogenic for young germfree pigs and caused septicemia and acute death within 48 h after intranasal inoculation (24). The MRP^- EF^- strains of type 1 were much less virulent but could still induce meningitis, polyarthritis, and occasionally death 7 to 10 days after inoculation (24). Taken together, these data indicate that the 110-kDa EF, possibly in conjunction with MRP or MRP^s, is associated with the pathogenicity of serotype 1 and 2 strains of *S. suis*.

Construction of mutant *S. suis* strains. To evaluate whether MRP, MRP^s, EF, and various EF* proteins play a critical role in the pathogenesis of *S. suis* infections, we constructed MRP^- EF^+, MRP^+ EF^-, and MRP^+ EF* mutants of *S. suis* type 2. Therefore, we electrotransformed the pathogenic MRP^+ EF^+ strain 10 (23) with nonreplicative plasmids in which the *mrp* or *epf* gene had been disrupted by insertion of an antibiotic resistance gene. To create pMR18 (18), the *mrp* gene in pMR11 (17) was disrupted by the insertion of the Spc^r gene from pDL282 (19). To construct pEF2-24, the internal 1,400-bp *Bgl*II-*Bam*HI fragment of the *epf* gene in pEF2-19 (16) was replaced by the Spc^r gene. For this purpose, we digested pEF2-19 with *Bgl*II and *Bam*HI. The largest fragment was blunt ended and ligated to the 1,200-bp blunt-ended fragment containing the Spc^r gene from pDL282 (19). The *epf* and Spc^r genes are transcribed in the same direction. For the construction of pEF2-37, we used a plasmid which contained the entire *epf** gene from strain 3921 (16). The plasmid was digested partially with *Sna*BI. Linearized fragments were purified and ligated to the 1,200-bp blunt-ended fragment containing the Spc^r gene.

Since Southern blotting and hybridization experiments showed that the *mrp* and *epf* genes of serotype 1 and 2 strains were highly homologous (results not shown), the same plasmids were used to construct the MRP^- EF^+ and MRP^s EF^- mutants of the pathogenic MRP^s EF^+ strain 6388 (24) of *S. suis* type 1.

pMR18, pEF2-24, pEF2-37, and pGA14-spc, a plasmid that is able to replicate in *S. suis* (9, 18), were used to electrotransform *S. suis* cells (18), and spectinomycin (100 μg/ml)-resistant

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colonies were selected. We obtained 10^6 (for type 2 cells) or 10^5 (for type 1 cells) transformants per μg of pGA14-spc DNA. After transformation with the nonreplicative plasmids pMR18, pEF2-24, and pEF2-37, we obtained 65 to 250 transformants per μg of DNA. We measured the production of MRP, EF, and EF* in a number of Spc^r transformants by using MRP- and EF-specific enzyme-linked immunosorbent assays (ELISAs) (22). In 5 to 23% of the Spc^r transformants, the production of MRP or EF was impaired or there had been a change to the production of EF* (data not shown). This suggests that in about 5 to 23% of the integration events, the original genes were replaced by the interrupted ones after a double-crossover recombination event.

To construct the MRP$^-$ EF$^-$ mutant of type 2, we electrotransformed an MRP$^-$ EF$^+$ mutant (10M7) with pEF2-28. To construct pEF2-28, the internal 1,400-bp *Bgl*II-*Bam*HI fragment of the *efp* gene in pEF2-19 (16) was replaced by the Erm^r gene from pNL9737 (12, 13). The *epf* and Erm^r genes are transcribed in the same direction. We obtained about 300 transformants per μg of DNA. As measured by MRP- and EF-specific ELISAs (22), about 5% of the Erm^r transformants produced neither MRP nor EF. This suggests that in 5% of the integration events the original *epf* gene was replaced by the interrupted gene.

Because serotype 1 strain 6388 has a natural resistance to erythromycin, we could not use pEF2-28 for the construction of an MRP$^-$ EF$^-$ derivative. Instead, we used pMR27 containing the *mrp* gene, which was disrupted by the insertion of a blasticidin resistance gene from pBEST402 (8). pBEST402 was digested with *Sma*I, and the 620-bp fragment was isolated and inserted into pMR11 (17) which had been partially digested with *Sca*I. In pMR27, the Brs^r and *mrp* genes are transcribed in the same direction. With pMR27, 20 blasticidin-resistant *S. suis* transformants were obtained per μg of DNA. As measured by MRP- and EF-specific ELISAs, seven of those transformants produced neither EF nor MRP. This indicated that in these transformants the *mrp* gene was replaced by the interrupted gene.

We analyzed the chromosomal DNA of the mutants by Southern blotting and hybridizations (10, 15). As expected, in the mutant strains, the original *mrp* and *epf* genes were replaced by the inactivated genes by double-crossover recombination events (results not shown). Moreover, in mutant 10E35*, the original *epf* gene was replaced by the *epf** gene.

Western blotting (immunoblotting) of proteins from mutant strains. By Western blot analysis (10, 15), we tested whether the MRP, MRP^s, EF, and EF* proteins were produced by the mutant strains. In these analyses, we used polyclonal antibodies directed against MRP and EF (22). The MRP$^-$ EF$^+$ mutant strains 10M7 and 6388M3 (Fig. 1, lanes 2 and 8) still produced EF but lacked MRP and MRP^s. Instead of MRP^s, mutant strain 6388M3 produced a 60-kDa protein. Since we had inserted the Spc^r gene 1,860 bp downstream of the translational start site of the *mrp* gene, an N-terminal 60 kDa of MRP was expected to be expressed. Surprisingly, mutant 10M7 did not produce the 60-kDa protein. The MRP$^+$ EF$^-$ mutant strains 10E125 and 6388E2 (Fig. 1, lanes 3 and 9) still produced MRP but lacked EF. Because in this case the Spc^r gene was inserted just downstream of the translational start site of the *epf* gene, residual EF products could not be detected. The MRP$^-$ EF$^-$ strains 10M7E26 and 6388E2M6 (Fig. 1, lanes 4 and 10) produce neither MRP nor EF. The MRP$^+$ EF* mutant strain 10E35* (Fig. 1, lane 6) produced MRP but lacked the 110-kDa EF protein. Instead, this strain produced an EF* protein of increased molecular mass.

Pathogenicity of mutant strains. We next tested the pathogenic properties of the wild-type and mutant strains of serotype 2 after experimental infection of newborn germfree pigs (20, 25). To predispose the pigs to infection with *S. suis*, 5-day-old pigs were inoculated intranasally with about 10^7 CFU of *Bordetella bronchiseptica* 92932. Two days later, the pigs were inoculated intranasally with *S. suis* type 2 (10^6 CFU). As a control, one group of pigs was inoculated with the nonvirulent MRP$^-$ EF$^-$ strain T15 (23). To score disease, we measured four different parameters: the body temperature of the pigs, the number of polymorphonuclear leukocytes (PMLs) in blood, clinical signs of disease, and colonization of the bacteria on various tissues.

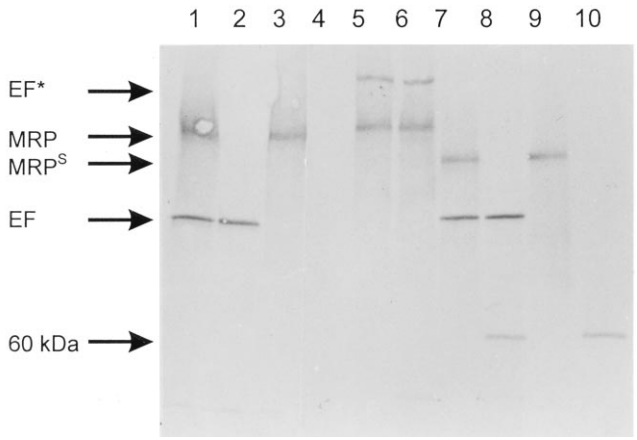

FIG. 1. Western blot of proteins present in the culture supernatants of the wild-type and mutant *S. suis* type 1 and 2 strains screened with anti-MRP and anti-EF polyclonal antibodies (22). Lane 1, wild-type serotype 2 strain 10; lane 2, mutant strain 10M7; lane 3, mutant strain 10E125; lane 4, mutant strain 10M7E26; lane 5, wild-type MRP$^+$ EF* strain 3921; lane 6, mutant strain 10E35*; lane 7, wild-type serotype 1 strain 6388; lane 8, mutant strain 6388M3; lane 9, mutant strain 6388E2; lane 10, mutant strain 6388E2M6.

The temperature of all pigs inoculated with the wild-type and mutant strains increased 2 days after inoculation with *S. suis* and remained high until the end of the experiment. We could not observe significant differences in the frequency of fever (i.e., the number of days during which pigs showed fever (>40°C) divided by the total number of days) between pigs inoculated with wild-type strains and those inoculated with mutant strains (Table 1). In contrast, the temperature of pigs inoculated with the negative control (strain T15) never exceeded 40°C.

In pigs inoculated with the wild-type or mutant type 2 strains, the number of PMLs increased ($>15 \times 10^9$ PMLs per liter) from day 2 onward. The percentage of blood samples with an increased number of PMLs did not differ significantly between pigs inoculated with the wild-type strain and those given the mutant strains (Table 1). In contrast, in pigs inoculated with strain T15, the frequency of samples with an increased number of PMLs was considerably lower.

We could observe specific as well as nonspecific signs of disease in all pigs inoculated with the wild-type or mutant strains (Table 1). All pigs inoculated with either wild-type or mutant strains died during the course of the experiment or were killed at a terminal stage of disease. The mortality rate induced by the wild-type and mutant *S. suis* strains was 100%. In contrast, all pigs inoculated with strain T15 remained clinically healthy until the end of the experiment (25 days).

In pigs inoculated with the wild-type or mutant type 2 strains, severe inflammations of the central nervous system,

TABLE 1. Virulence of wild-type and mutant *S. suis* strains in pigs by various disease parameters

S. suis strain	Serotype	Phenotype	No. of pigs	Survival[a]	Frequency of fever[b]	Frequency of leukocytosis[c]	Frequency of clinical signs[d]		Frequency of colonization of:		
							Specific[e]	Nonspecific[f]	CNS[g]	Serosae[g]	Joints[h]
10	2	MRP^+ EF^+	4	11	46	38	42	40	50	0	21
10M7	2	MRP^- EF^+	4	14	33	63	22	39	75	63	15
10E125	2	MRP^+ EF^-	5	12	45	56	33	36	100	60	8
10M7E26	2	MRP^- EF^-	4	11	45	59	22	37	100	88	2
10E35*	2	MRP^+ EF*	4	11	33	53	30	30	80	38	6
T15	2	MRP^- EF^-	4	25	0.6	17	0	0	0	0	0
6388	1	MRP^s EF^+	4	2	28	0	0	16	100	88	71
6388E2M6	1	MRP^- EF^-	16	2	21	4	6	3	88	66	49
6555	1	MRP^- EF^-	4	14	6	0	3	4	100	63	67

[a] The average number of days that pigs survived after infection.
[b] The number of days during which pigs showed fever (>40°C) divided by the total number of days.
[c] The number of blood samples with $>15 \times 10^9$ PMLs per liter divided by the total number of samples.
[d] The number of days during which pigs showed clinical signs of disease divided by the total number of days.
[e] Lameness, nervous disorders.
[f] Lack of appetite, depression.
[g] The number of pigs from which *S. suis* was isolated postmortem divided by the total number of pigs.
[h] The number of positive joints divided by the total number of joints per group.

serosae, and joints were detected. Pigs inoculated with strain T15 had virtually no pathological changes (Table 1).

S. suis strains and *B. bronchiseptica* could be isolated from the nasopharynx and feces of all pigs from 1 day postinfection until the end of the experiment. Postmortem, wild-type as well as mutant *S. suis* strains could be isolated from the central nervous system (CNS), the serosae, and the joints (Table 1). Strain T15 could be isolated only from the lungs (results not shown). We tested the expression of MRP, EF, and EF* in the *S. suis* strains, reisolated from the CNS, by ELISAs and Western blot analysis. Moreover, we examined the chromosomal structures of the reisolated strains by Southern blotting and hybridization. The data showed that all *S. suis* strains isolated from the CNS still had the original genotype and phenotype (results not shown).

We also compared the pathogenicity of the wild-type strain 6388 of serotype 1 with that of the isogenic MRP^- EF^- mutant strain (6388E2M6) and that of a serotype 1 MRP^- EF^- field strain (6555 [24]). Because wild-type strain 6388 is very pathogenic for young pigs, the pigs were inoculated intranasally with 10^4 CFU. No significant differences in pathogenicity between the wild-type and the mutant strains could be observed. All pigs inoculated with the wild-type and mutant strains died within 2 days after infection or were killed at a terminal stage of disease (Table 1). Although the serotype 1 MRP^- EF^- field strain 6555 caused disease in pigs, this strain was less virulent. Pigs inoculated with strain 6555 died or had to be killed on day 14 at a terminal stage of disease.

Taken together, these data showed that mutant strains of *S. suis* types 1 and 2, impaired in the expression of MRP and EF or producing an EF* protein, can induce disease in pigs. Therefore, MRP, MRP^s, EF, and EF* are not required for causing disease. This could suggest that, in contrast to our previous ideas, MRP, MRP^s, EF, and EF* themselves do not contribute to the pathogenicity of *S. suis* type 1 and 2 infections, but the synthesis of these proteins is coincidentally associated with pathogenicity. An alternative explanation, however, is that the virulence associated with *S. suis* infections is a multifactorial process in which particular functions can be fullfilled by redundant or alternative factors. This would mean that in the absence of MRP and EF, other virulence factors can take over their functions. If this is true, it could explain why *S. suis* strains impaired in the expression of MRP and EF are as virulent as the wild-type strains. Redundancy of virulence factors has been observed before (6). The flagella and fimbriae of *Salmonella typhimurium* (11), as well as the filamenous hemagglutinin and pertussis toxin of *Bordetella pertussis* (14), seem to be redundant virulence factors (6). Since it is known that the virulence of various streptococci is a complex, multifactorial process (2, 4), the involvement of a variety of different virulence factors in the pathogenicity of *S. suis* infections should be envisaged.

To construct the MRP^- mutants, the *spc* gene was inserted 1,860 bp downstream of the translational start site of the *mrp* gene. As a result, the mutant strains were expected to produce the N-terminal 60 kDa of MRP. Mutant strain 10M7 did not produce this protein. We do not know the reason for this observation. A possible explanation is that mutations in the gene that were not detected by Southern hybridizations prevented the production of the protein. In contrast to MRP and MRP^s, which are associated with the cellular membrane, the 60-kDa protein is secreted into the culture supernatant. If in the mutant 6388M3 the 60-kDa protein still can fulfill (part of) the function of MRP^s, this could explain the pathogenicity of this mutant.

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