High-efficiency transformation and gene inactivation in *Streptococcus suis* type 2

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An efficient electrotransformation system for *Streptococcus suis* type 2 is described. It is demonstrated that vectors based on the broad-host-range plasmid pWVO1 replicate in *S. suis* type 2. Transformation efficiencies of about 10⁷ transformants per µg of plasmid DNA could be obtained. Derivatives of plasmid pBR322 containing *S. suis* chromosomal DNA did not replicate but integrated into the chromosome. Southern hybridization analysis revealed that double as well as single cross-over integration events had occurred. Double cross-over events occurred at a frequency of about 15%. With these transformation and integration systems, recombinant DNA technology can now be applied to this important pathogenic species.

Keywords: transformation, homologous recombination, double and single cross-over integrations, gene amplification

INTRODUCTION

Streptococcus suis type 2 is an important cause of meningitis, septicaemia, arthritis and sudden death in young pigs (Clifton-Hadley, 1983; Vecht *et al.*, 1985), and of meningitis in humans (Arends & Zanen, 1988). During the last few years *S. suis* type 2 infections have become a major problem in almost all countries with an intensive pig industry. So far, little is known about the pathogenesis and the epidemiology of *S. suis* type 2 infections.

Strains of *S. suis* type 2 may differ in virulence (Vecht *et al.*, 1989). We previously identified two proteins, muramidase-released protein (MRP) and extracellular factor (EF), of *S. suis* type 2 that seem to be associated with pathogenicity (Vecht *et al.*, 1991). To study the role of MRP and EF in the pathogenesis of *S. suis* type 2 infections, isogenic mutants impaired in the expression of MRP and EF could be of great benefit. To construct these mutants, a system for the efficient introduction of exogenous DNA into *S. suis* type 2 is required. Moreover, the expression of an antibiotic-resistance gene that is suitable for selection of transformants and homologous recombination in *S. suis* type 2 is necessary. So far, transformation systems and gene replacements have not been described for this organism. At present, electrotransformation is the system most widely used to efficiently introduce DNA into

Gram-negative (Dower *et al.*, 1988; Miller *et al.*, 1988; Sreenivasan *et al.*, 1991), and Gram-positive bacteria (Van der Lelie *et al.*, 1988; Holo & Nes, 1989; Dunny *et al.*, 1991). In the present paper we show that electrotransformation can also be used to efficiently introduce plasmid DNA into *S. suis* type 2. To develop the electroporation system, plasmid vectors which replicate in *S. suis* were required. Here, we report that plasmids based on the broad-host-range plasmid pWVO1 replicate in *S. suis*. In addition, by using non-replicative plasmids, chromosomal integration and double cross-over recombination events were obtained.

METHODS

Bacterial strains and growth conditions. The virulent strains D282 and 10 of *S. suis* type 2 (Vecht *et al.*, 1989, 1992) were used as parent strains for the construction of the mutants. *Escherichia coli* strain JM101 (Messing, 1979) was used as host for recombinant plasmids. *S. suis* strains were grown in Todd-Hewitt broth (code CM189, Oxoid), and plated on Columbia agar blood base (code CM331, Oxoid) containing 6% (v/v) horse blood. Antibiotics were added to the plates at the following concentrations: chloramphenicol, 5 µg ml⁻¹; erythromycin, 1 µg ml⁻¹; and spectinomycin, 100 µg ml⁻¹. *E. coli* strains were grown in Luria broth (Miller, 1972) and plated on Luria broth containing 1.5% (w/v) agar. Antibiotics were added to *E. coli* cultures or plates at the following concentrations: ampicillin, 50 µg ml⁻¹; chloramphenicol, 5 µg ml⁻¹; erythromycin, 100 µg ml⁻¹; and spectinomycin, 50 µg ml⁻¹.

Plasmids. The plasmids used in this study are shown in Table 1.

Abbreviations: EF, extracellular factor; MRP, muramidase-released protein.

Table 1. Plasmids

Plasmid	Relevant properties*	Source or reference
pWVO1	Cryptic plasmid of <i>Lactococcus lactis</i> subsp. <i>cremoris</i> Wg2	Kok <i>et al.</i> (1984)
pGL3	Replication functions of pWVO1, Cm ^R Km ^R	Kindly provided by J. Kok
pGA14	Replication functions of pWVO1, Em ^R	Perez-Martinez <i>et al.</i> (1992)
pGA14-spc	Replication functions of pWVO1, Em ^R Spc ^R	This work
pKUN19	Replication functions of pUC, Amp ^R	Konings <i>et al.</i> (1987)
pKUN19-spc	Replication functions of pUC, Amp ^R Spc ^R	This work
pMR11	pKUN19 containing the entire <i>mrp</i> gene	Smith <i>et al.</i> (1992)
pMR17	pMR11 with the <i>spc</i> ^R gene inserted into the <i>ScaI</i> site of the <i>mrp</i> gene	This work
pMR18	pMR11 with the <i>spc</i> ^R gene inserted into the <i>ScaI</i> site of the <i>mrp</i> gene	This work
pDL282	Replication functions of pBR322 and pVT736-1, Amp ^R Spc ^R	Sreenivasan <i>et al.</i> (1991)

* Amp^R, ampicillin resistant; Cm^R, chloramphenicol resistant; Em^R, erythromycin resistant; Spc^R, spectinomycin resistant.

DNA manipulations were performed by standard techniques (Sambrook *et al.*, 1989). pGL3 contains the replication functions of pWVO1 (Kok *et al.*, 1984), the *alacZ* gene of M13mp10 (Messing, 1983), the chloramphenicol-resistance gene of pC194 (Horinouchi & Weisblum, 1982) and a kanamycin-resistance gene of *Enterococcus faecalis* (Trieu-Cuot & Courvalin, 1983). To disrupt the *mrp* gene in pMR11 (Smith *et al.*, 1992), the *spc*^R gene from pDL282 (Sreenivasan *et al.*, 1991) was inserted into the *ScaI* site within the *mrp* gene. Therefore, we digested pDL282 with *HindIII* and partially with *KpnI*. The 1200 bp *HindIII*–*KpnI* fragment containing the *spc*^R gene was isolated. The restriction sites were filled in with Klenow enzyme and the fragment was ligated to pMR11, which was partially digested with *ScaI*. In pMR18, the *mrp* and the *spc*^R genes are transcribed in the same direction (see Fig. 1a), whereas in pMR17 they are transcribed in opposite directions (Fig. 1b). In pKUN19-spc the 1200 bp *HindIII*–*KpnI* fragment containing the *spc*^R gene was ligated to *HindIII*- and *KpnI*-digested pKUN19. To construct pGA14-spc, we ligated the 1200 bp *HindIII*–*KpnI* fragment containing the *spc*^R gene to the largest *HindIII*–*KpnI* fragment of pGA14 (Perez-Martinez *et al.*, 1992), which contained the replication functions. Ligation mixtures were used to transform *E. coli* JM101 and spectinomycin-resistant colonies were selected.

Electrotransformation of *S. suis* type 2. Overnight cultures in Todd–Hewitt broth were diluted 50-fold in 200 ml fresh Todd–Hewitt broth supplemented with 40 mM DL-threonine. Cultures were incubated for a further 3 h at 37 °C to an OD₆₀₀ of about 0.4. Cells were then harvested by centrifugation and washed twice with 20 ml ice-cold double-distilled water, twice with 20 ml ice-cold 0.3 M sucrose and once with 20 ml ice-cold 0.3 M sucrose plus 15% (v/v) glycerol. The cells were re-suspended in 1 ml 0.3 M sucrose plus 15% glycerol and were either used directly for electrotransformation or stored at –80 °C. Portions (50 µl) of these cell suspensions were mixed with 5 µg plasmid DNA at 0 °C. The mixtures were transferred into prechilled sterile Gene Pulser cuvettes (inter-electrode distance 0.2 cm; Bio-Rad). Electrotransformations were done using the Bio-Rad Gene Pulser apparatus. Pulses were achieved with a setting of 25 µF, 2.5 kV and 200 Ω. This setting resulted in a time constant ranging from 4.6 to 5.2 ms. After the electric pulse the cells were diluted immediately in 1 ml Todd–Hewitt broth plus 0.3 M sucrose and incubated for 2 h at 37 °C. They were then plated on agar plates containing selective antibiotics.

Detection of MRP and EF by Western blotting and ELISA. For Western blot analysis, proteins were separated by SDS-PAGE

(Laemmli, 1970) with 4% stacking gels and 6% separating gels. The separated proteins were transferred to nitrocellulose in a Semi-Dry transfer cell (Bio-Rad). To detect specific proteins the blots were incubated with polyclonal antibodies directed against MRP and EF (Vecht *et al.*, 1991). Bound antibodies were visualized with anti-rabbit sera conjugated with alkaline phosphatase (Zymed Laboratories) as described by Sambrook *et al.* (1989).

The presence of MRP and EF in *S. suis* culture supernatants was determined with MRP- and EF-specific double-antibody sandwich ELISAs as described by Vecht *et al.* (1993).

Southern blotting and hybridization. Chromosomal DNA was isolated as described by Sambrook *et al.* (1989). DNA fragments were separated on 0.8% agarose gels and transferred to Zeta-Probe GT membranes (Bio-Rad) as described by Sambrook *et al.* (1989). DNA probes were labelled with [α -³²P]dCTP (3000 Ci mmol⁻¹, 111 TBq mmol⁻¹; Amersham) by use of a random-primed labelling kit (Boehringer). The DNA on the blots was hybridized at 65 °C with DNA probes as recommended by the supplier of the Zeta-Probe membranes. After hybridization, the membranes were washed twice with a solution of 40 mM sodium phosphate, pH 7.2, 1 mM EDTA, 5% (w/v) SDS for 30 min at 65 °C and twice with a solution of 40 mM sodium phosphate, pH 7.2, 1 mM EDTA, 1% SDS for 30 min at 65 °C.

RESULTS

Electrotransformation of *S. suis* type 2

In order to develop an electrotransformation system for *S. suis* type 2, plasmids which replicate in *S. suis* type 2 were required. Since such plasmids were not available we tested whether pWVO1-based-host-range plasmids could be used. Plasmid pWVO1 was originally isolated from *Lactococcus lactis* subsp. *cremoris* Wg2 (Kok *et al.*, 1984) and is able to replicate in *E. coli* as well as in a considerable number of Gram-positive bacteria (Bron, 1990; Kok, 1991). Therefore we assumed that pWVO1-based plasmids may also replicate in *S. suis*. Because the virulent *S. suis* type 2 strains D282 and 10 are sensitive to both chloramphenicol and spectinomycin, and strain 10 is sensitive to erythromycin as well, we used the plasmids pGL3, pGA14 and pGA14-spc (Table 1) in our initial

Table 2. Electrotransformation frequencies

<i>S. suis</i> strain	Electrotransformation frequency*				
	pGL3 (Cm ^R)	pGA14 (Em ^R)	pGA14-spc (Spc ^R)	pKUN19-spc (Spc ^R)	No DNA† (Spc ^R)
D282	1.5 × 10 ⁴	ND	10 ⁷	30	30
10	60	2.5 × 10 ⁴	3 × 10 ⁶	20	20

* Number of transformants per µg DNA. The numbers represent data of one representative experiment.
 ND, Not determined.

† Number of colonies derived from a control transformation experiment.

Table 3. Electrotransformation of *S. suis* type 2 strains with non-replicative plasmids

<i>S. suis</i> strain	Plasmid	Electrotransformation frequency*	No. of Spc ^R tfm. tested for MRP production	No. of MRP ⁻ Spc ^R tfm.
D282	pGA14-spc	3 × 10 ⁶		
10	pGA14-spc	1 × 10 ⁶		
D282	pKUN19-spc	30		
10	pKUN19-spc	20		
D282	pMR17	450	36	5 (13.8%)
10	pMR17	ND		
D282	pMR18	500	72	12 (16.7%)
10	pMR18	250	72	12 (16.7%)

* Number of transformants (tfm.) per µg DNA. The numbers represent data of one representative experiment. ND, Not done.

experiments. The plasmids were isolated from *E. coli* and subsequently used for electrotransformation of strains D282 and 10. Chloramphenicol- and erythromycin-resistant transformants were obtained at a low frequency after incubating the plates for 40 h at 37 °C (Table 2). Spectinomycin-resistant transformants, however, were obtained after 16 h at 37 °C. Moreover, the latter transformants occurred at a high frequency (Table 2). We confirmed the presence of the donor plasmids in the transformants by DNA isolation and Southern hybridization (results not shown). These data show that *S. suis* type 2 cells can be electrotransformed at a high frequency, that plasmids derived from pWVO1 replicate in *S. suis* type 2 and that the spectinomycin-resistance gene from *Enterococcus faecalis* is a suitable selectable marker for *S. suis* type 2. In contrast to the pWVO1-derived plasmids, plasmids based on the replication functions of pUC did not produce spectinomycin-resistant colonies above background levels (Table 2).

To obtain optimal electrotransformation efficiencies we varied conditions for growth of the cells, washing procedures and the electric field strength. None of these variations increased the transformation efficiencies substantially. In a number of streptococcal species, the transformation efficiency was significantly increased when

the cells were grown in the presence of glycine (Holo & Nes, 1989; Dunny *et al.*, 1991). The growth of *S. suis* cells, however, was strongly inhibited by glycine (concentrations > 0.25%). In addition, the cells frequently lysed during electrotransformation when grown with glycine. If the cells were stored at -80 °C prior to the electrotransformation, no significant effect on the transformation efficiency was observed. Therefore, we could use frozen electro-competent cells from one batch in successive experiments.

Chromosomal integration

To study whether pUC-based plasmids could be used to obtain chromosomal integrations in *S. suis* type 2, we constructed plasmids in which the *mrp* gene of *S. suis* was disrupted by the insertion of the *spc^R* gene (LeBlanc *et al.*, 1991; Sreenivasan *et al.*, 1991). We previously described the cloning of the *mrp* gene into the pUC-derived plasmid vector pKUN19 (Smith *et al.*, 1992). In pMR18 the disrupted *mrp* and the *spc^R* gene are transcribed in the same direction. In pMR17 these genes are transcribed in opposite directions. We electrotransformed strains D282 and 10 (MRP⁺ EF⁺) with pMR17 and pMR18. To determine the electrocompetence of the cells, we also transformed them with pGA14-spc, a plasmid that is able

Fig. 1. Schematic representation of the chromosomal structures after double (a) and single (b) cross-over recombination events between pMR18 and pMR17 and the chromosome of *S. suis* type 2 strain D282. (c) Schematic representation of the amplification due to the 1990 bp repeats and of the deletion of the intact copy of the *mrp* gene by recombination between the 2840-bp repeats. ■, Chromosomal *S. suis* type 2 DNA containing the *mrp* gene; ■, *spc*^R gene; —, pKUN19 DNA; ~, chromosome of *S. suis* type 2. C, *Clal*; E, *EcoRI*; H, *HindIII*; S, *SalI*; X, *XbaI*. The sizes of the *EcoRI*-*Clal* and *SalI* fragments are indicated in bp.

to replicate in *S. suis*. With strain D282 we obtained 3×10^6 transformants per μg pGA14-*spc* DNA and with strain 10 we obtained 1×10^6 transformants per μg pGA14-*spc* DNA (Table 3). After transformation with the non-replicative plasmids pMR17 and pMR18 we obtained 250–500 transformants per μg DNA (Table 3). In transformations with pMR18, transformants were obtained after a 16 h incubation period at 37 °C. In transformations with pMR17, however, transformants were obtained after 40 h at 37 °C. Only low numbers of colony-forming units were obtained after transformation of *S. suis* cells with pKUN19-*spc*, a plasmid that is unable to replicate in *S. suis* and does not contain chromosomal *S. suis* sequences (Table 3). Based on these data we surmised

that the *Spc*^R transformants obtained with pMR17 and pMR18 were the result of integration of the plasmid into the chromosome by homologous recombination. Two classes of *Spc*^R recombinants can be envisaged (Leenhouts *et al.*, 1990, 1991; Fig. 1): a double cross-over recombination event (Fig. 1a) is expected to produce an MRP⁻ phenotype, and plasmid integration via a single cross-over recombination (Fig. 1b) is expected to result in the original MRP⁺ phenotype (the entire *mrp* gene and an inactivated *mrp* gene are present). To differentiate between these two types of recombinants, we measured the production of MRP in a number of *Spc*^R transformants by means of an MRP-specific ELISA (Vecht *et al.*, 1993). As a control we measured the production of EF by an EF-

Fig. 2. Southern blots of chromosomal DNA of *S. suis* strain D282 and various MRP⁻ strains obtained after transformation of strain D282 with pMR18. Chromosomal DNA was digested with *Eco*RI and *Cla*I. The probes used were: (a) a 1200 bp *Kpn*I-*Hind*III fragment containing the *spc*^R gene, (b) an internal 1210 bp *Xba*I-*Sca*I fragment of the *mrp* gene, and (c) pKUN19. Strain designations are indicated above the lanes.

specific ELISA. As expected, all Spc^R transformants produced EF. In contrast, about 15% of the Spc^R transformants tested did not produce MRP (Table 3). This suggests that in about 15% of the integrations the original *mrp* gene was replaced by the interrupted *mrp* gene.

Characterization of mutants by Southern hybridization

We analysed the chromosomal structure of a number of Spc^R MRP⁻ mutants by Southern hybridization. Fig. 2 shows the results of Southern hybridization with four Spc^R MRP⁻ mutants that were obtained after transformation of strain D282 with pMR18. Chromosomal DNA isolated from the mutant strains and the recipient strain D282 was digested with *Eco*RI and *Cla*I. After electrophoresis, these samples were hybridized with three different probes: a fragment containing the *spc*^R gene, an *Xba*I-*Sca*I fragment located within the coding region of the *mrp* gene, and plasmid pKUN19. As expected, DNA of the four transformants hybridized with the *spc*^R gene (Fig. 2a). In strain D282 a 2840 bp *Eco*RI-*Cla*I fragment hybridized with the *mrp* probe (Fig. 2b). This fragment was absent in the mutant strains DM13 and DM18. In these two mutants the *mrp* probe hybridized with a 4040 bp *Eco*RI-*Cla*I fragment. This fragment also hybridized with the *spc*^R gene probe (compare Figs 2a and 2b). Strains DM13 and DM18 did not hybridize with pKUN19 (Fig. 2c), and therefore did not contain vector sequences. These results strongly suggest that in DM13 and DM18 the *mrp* gene was replaced by the inactivated

mrp gene via a double cross-over recombination event (Fig. 1a).

In the digests of mutants DM8 and DM20 the *mrp* probe hybridized with both the 2840 bp and the 4040 bp *Eco*RI-*Cla*I fragments (Fig. 2b). Apparently, these mutants contain a copy of the original *mrp* gene as well as a copy of the inactivated *mrp* gene. Moreover, in strains DM8 and DM20 a 4970 bp and a 820 bp *Eco*RI-*Cla*I fragment hybridized with pKUN19 (Figs 1 and 2c). This indicates that these strains also contained vector sequences. These data indicate that in strains DM8 and DM20 the integration of the plasmid into the chromosome occurred by a single cross-over recombination event (Fig. 1b).

We also analysed the chromosomal structure of four Spc^R MRP⁻ mutants obtained after electrotransformation of strain D282 with pMR17. As expected, DNA from all four mutant strains hybridized with the *spc*^R gene (data not shown). DNA from strain D282 contained the 2840 bp *Eco*RI-*Cla*I fragment that hybridized with the *mrp* probe (Fig. 3a). In the four mutant strains this 2840 bp hybridizing fragment was absent, and was replaced by the 4040 bp fragment. These data suggest that in all the mutant strains the original *mrp* gene was replaced by the interrupted *mrp* gene. However, the intensities of the hybridizing signal differed remarkably between the parent and the mutant strains. Since we applied identical amounts of DNA to the agarose gel (Fig. 3c), we concluded that the mutant strains contained more than one copy of the interrupted *mrp* gene. One possible explanation for this is that pMR17 was initially integrated

Fig. 3. Southern blots and agarose gels of chromosomal DNA of *S. suis* strain D282 and various MRP⁻ strains obtained after transformation of strain D282 with pMR17. (a) Southern blot of the gel shown in (c), hybridized with a ³²P-labelled 1210 bp *Xba*I-*Sca*I fragment of the *mrp* gene. (b) Southern blot of chromosomal DNA digested with *Sca*I and probed with ³²P-labelled pKUN19. (c) DNA samples digested with *Eco*RI and *Cla*I on a 0.8% agarose gel. (d) Lane 1, molecular size markers (lambda DNA digested with *Hind*III); lane 2, uncut DNA from strain DM14 on a 0.8% agarose gel. (e) Southern blot of chromosomal DNA digested with *Bgl*II and hybridized with a ³²P-labelled 1210 bp *Xba*I-*Sca*I fragment of the *mrp* gene. Strain designations are indicated above the lanes.

by a single cross-over recombination and that the integrated plasmid was subsequently amplified (Fig. 1b, c). Another possibility is that a multimeric form of the plasmid was integrated in the chromosome by a single cross-over recombination event. After integration of the plasmid as indicated in Fig. 1(b), amplification is possible due to the presence of the 1990 bp repeats which flank the integrated plasmid (Jannire *et al.*, 1985; Leenhouts *et al.*, 1990; Fig. 1c). After amplification the chromosome will contain multiple copies of the 4970 bp, 4040 bp and 820 bp *Eco*RI-*Cla*I fragments (Fig. 1c). Amplification of these fragments was visible directly on ethidium-bromide-stained agarose gels (Fig. 3c). Integration of the entire plasmid in the chromosome was supported by the observation that in all mutant strains a 9830 bp *Sca*I fragment strongly hybridized with vector sequences (Figs. 1c and 3b). Moreover, freely replicating plasmid was not visible in a sample of uncut DNA (Fig. 3d). We also analysed integration of the plasmid in the chromosome after digesting the chromosomal DNA with *Bgl*II. This enzyme does not cut within pMR17 nor within the *mrp* gene. DNA from strain D282 contained a *Bgl*II fragment of about 12 kb that hybridized with the *mrp* probe (Fig. 3e). In the mutant strains this fragment was replaced by a larger hybridizing fragment. This strongly indicates that in the mutant strains the plasmid is integrated in the chromosome. All MRP⁻ strains lack the original copy of the *mrp* gene. This indicates that in these strains the original *mrp* gene was deleted. Recombination between the 2840 bp repeats could explain this event (Fig. 1c).

Characterization of mutants by Western blotting

We tested whether the MRP and EF proteins were present in the mutant strains by Western immunoblotting. All MRP⁻ strains still produced EF, but lacked the 136 kDa MRP (Fig. 4). Some of the mutant strains (DM8, DM18, DM20, DM24 and DM16) produced a 60 kDa immuno-reactive protein. Because we inserted the *spc*^R gene into the *mrp* gene 1860 bp downstream from the translational start site, the N-terminal 60 kDa of MRP was expected to be expressed. Surprisingly, the other mutants (DM13, DM14 and DM29) did not produce the 60 kDa protein. Apparently, expression of the truncated protein was prevented in these strains.

DISCUSSION

In this paper we describe the introduction of exogenous DNA into *S. suis* type 2 by electroporation. With the parameters described, we obtained a transformation efficiency of about 10⁷ transformants per µg DNA. Before this, efficient transformation had not been described for *S. suis*. Recently, conjugational transfer of antibiotic-resistance markers between *S. suis* strains was reported (Stuart *et al.*, 1992). The efficiency of transfer, however, was very low.

Also, plasmids replicating in *S. suis* had not previously been described. We electroporated *S. suis* cells with the pWVO1-based broad-host-range vectors and demonstrated that they are able to replicate in *S. suis* type 2.

Fig. 4. Western blots of proteins present in culture supernatants of *S. suis* type 2 strain D282 and several MRP⁻ mutants screened with anti-MRP and anti-EF polyclonal antibodies (Vecht *et al.*, 1993).

pWVO1 was originally isolated from *Lactococcus lactis* subsp. *cremoris* Wg2 (Kok *et al.*, 1984). The plasmid replicates in a large variety of Gram-positive bacteria, including all species of lactic acid bacteria, various streptococci, several *Bacillus* species, *Enterococcus faecalis*, *Staphylococcus aureus* (Kok *et al.*, 1984; Kok, 1991) and *Clostridium acetobutylicum* (Williams *et al.*, 1990). It also replicates in *E. coli* (Bron, 1990; Kok *et al.*, 1984). During the last few years, a series of small versatile pWVO1-derived vectors has been constructed (Kok, 1991). Because these plasmids also replicate in *S. suis* type 2, recombinant DNA technology can now easily be applied to this organism.

In the present studies we have also inactivated the *mrp* gene in the chromosome of *S. suis* type 2. The protein encoded by this gene was previously shown to be associated with virulence of *S. suis* type 2 for young pigs (Vecht *et al.*, 1991, 1992). Double as well as single cross-over integrations were obtained. The type of integrant obtained was apparently affected by the level of expression of the *spc^R* gene. Double cross-over recombination events were found only if we used plasmids in which the *spc^R* gene and the gene in which it was inserted were transcribed in the same direction. A likely explanation for this observation is that in this case the expression of a single copy of the *spc^R* gene was increased to a level sufficient to cause resistance to spectinomycin. In contrast, if the *spc^R* gene and the gene in which it was inserted were transcribed in opposite directions, we found that the mutant strains contained multiple copies of the inactivated

gene in the chromosome. Clearly, in this case the amount of the *spc^R* gene product was increased by amplification. If we used plasmids in which the *spc^R* gene and the *mrp* gene were transcribed in opposite directions, transformants were only obtained after an incubation period of 40 h at 37 °C. Therefore, the slow growth of the transformants seems to be associated with amplification of the *spc^R* gene product, which is necessary to enable the cells to grow on selective plates. Amplification is commonly observed after integration of plasmid DNA via a single cross-over event (Jannire *et al.*, 1985; Leenhouts *et al.*, 1990). After integration of plasmids by single cross-over recombination, the chromosome will initially contain the inactivated copy as well as the original copy of the gene. Most of our mutant strains, however, lacked the original copy of the gene. Therefore, those mutants could be the result of a single cross-over integration and subsequent amplification which was followed by deletion of the original gene. This deletion most probably resulted from recombination between the repeats flanking this gene. Alternatively, these mutant strains could be the result of a double cross-over recombination event with a multimeric plasmid. This will result in the deletion of the original gene and can be followed by amplification of the inactivated genes.

Although they did not produce MRP, some mutant strains (DM8, DM20) still seemed to contain a copy of the original *mrp* gene. We do not know the reason for this observation. A possible explanation is that mutations in the gene, not detectable by Southern hybridizations, prevented the production of the proteins. A second mutation could also explain the fact that mutants DM13, DM14 and DM29 did not produce the N-terminal 60 kDa part of the MRP protein, which was expected to be synthesized by the inactivated *mrp* genes.

With the availability of efficient transformation, chromosomal integration and gene replacement systems in *S. suis* type 2, the role of potential virulence factors in the pathogenesis of this organism can be studied. More specifically, in addition to the MRP⁻ mutants, we will construct EF⁻ mutants and MRP⁻ EF⁻ mutants and test their virulence after experimental infections in pigs. This should answer the question whether MRP and EF play a role in the pathogenesis of *S. suis* type 2.

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