# Construction of the Fusion Protoplast of Swine Escherichia coli with Hemophilus parasuis 

Chen Rui-Ai, Tang Xu, Wang Wenhao, Liu Haopeng, Zhang Xianhao and He Dongsheng<br>Ministry of Agriculture, Key Laboratory of Biotechnology and Bioproducts Development for Animal Epidemic Prevention,<br>South China Agricultural University, 510642 Guangdong, China


#### Abstract

This is the first study to construct successfully a hybrid protoplast of swine Escherichia coli (sw E. coli) E2010 with Hemophilus parasuis (Hps) H2011 strain. By conventional methods two types of bacteria, identified by specific drug resistant and rabbit-anti antibodies had been fused to construct a fusion protoplast named EH1011. EH1011 showed their parental drug resistance and agglutination identity by rabbit anti-E2010 serum and the rabbit anti-H2011 serum. The genetic characteristics of the fusion strains are stable within 15 generations through the identification of colony morphology, germ staining characteristics, biochemical characteristics and direct agglutination test. This fusion protoplast of swine E. coli and Hps has laid a foundation for the development of a new type of swine bivalent attenuated vaccine to control swine Escherichia coli and Hemophilus parasuis.


Key words: Swine Escherichia coli, Hemophilus parasuis, protoplast fusion, vaccine, genetic

## INTRODUCTION

The diseases caused by E. coli or Haemophilus parasuis has been bringing serious harm to the global pig industry and a great economic loss (Kielstein and Rapp-Gabrielson, 1992; Rafiee and Blackall, 2000; Vahle et al., 1995). It needs addressing urgently for us to control the two diseases as soon as possible.

Classical methods for control of the two diseases have relied upon either antimicrobial drugs for treatment of the diseases. Vaccines (inactivated vaccines and subunit vaccines) may play an important role in the prevention of the diseases. Traditional methods of prevention and treatment have their own limitations: antibiotics are likely to generate drug-resistant bacterias and negative impact on health of humans and animals. In order to overcome the short period of inactivated vaccine protection, the booster vaccination is necessary but this may cause the stress to pigs. Subunit vaccines may not develop good cellular immunity. On the contrary, attenuated vaccines known as live vaccines may provide alternative way to control the bacterial diseases. Attenuated vaccine has many advantages for example, less immunization dose, minor side effects and long lasting immunity, etc.

Protoplast fusion also known as cell fusion, cell hybridization or somatic hybridization was developed by gene recombination and breeding. In recent years, the technology was widely used in many fields (Bradshaw, 2006; Chen et al., 2006) and has made lots of pioneering research in the fields of agriculture, environmental protection, medicine and preventive veterinary science. It has important significance and value that a heterozygous strains of different kinds of bacteria can be constructed with microbial protoplast fusion technology for prevention and treatment of major epidemics.

## MATERIALS AND METHODS

Bacteria strains: Swine Escherichia coli E2010, serotped $\mathrm{O}_{107}$ with resistance to amikacin sulfate and Hemophilus parasuis H 2011 with resistance to rifampicin which were obtained from College of Veterinary Medicine of South China Agricultural University were used as parents strains in the protoplast fusion experiments.

Protoplast formation and regeneration: The method of protoplasts preparation was changed according to described previously (Weiss, 1976). Strains E2010,

[^0]stocked in the $\operatorname{TSB}\left(17 \mathrm{~g} \mathrm{~L}^{-1}\right.$ tryptone, $3 \mathrm{~g} \mathrm{~L} \mathrm{~L}^{-1}$ multivalent peptone $3 \mathrm{~g}, 5 \mathrm{~g} \mathrm{~L}^{-1} \mathrm{NaCl}, 2.5 \mathrm{~g} \mathrm{~L}^{-1} \mathrm{~K}_{2} \mathrm{HPO}_{4}, 2.5 \mathrm{~g} \mathrm{~L}^{-1}$ glucose) medium was cultured at $37^{\circ} \mathrm{C}$ for 5 h on a shaker in the TSB medium. The 4 mL well-grown cells were precipitated by $4,000 \times \mathrm{g}$ centrifugation for 20 min at room temperature and washed repeatedly twice with 0.01 M Tris buffer. Cells were resuspended by 4 mL hypertonic tris buffer ( $0.01 \mathrm{~mol} \mathrm{~L}^{-1}$ Tris, $0.5 \mathrm{~mol} \mathrm{~L}^{-1}$ sucrose, $0.01 \mathrm{molL}^{-1}$ $\mathrm{CaCl}_{2}, \mathrm{pH} 7.0$ ) and preheated lysozyme was added in at series final concentration as $0.05,0.1,0.2,0.4 \mathrm{mg} \mathrm{mL}^{-1}$. The cell suspension mixed with lysozyme were incubated in $37^{\circ} \mathrm{C}$ water bath for $10,15,20,25$ or 30 min , respectively. Then, potassium Ethylene Diamine Tetraacetate (EDTA, $0.1 \mathrm{~mol} \mathrm{~L}{ }^{-1}, \mathrm{pH} 8.0$ ) was added slowly to the mixture at final concentration of 0.01 M and was placed in a $37^{\circ} \mathrm{C}$ water bath for 15 min . Centrifuged at $3500 \times \mathrm{g}$ for 20 min at $4^{\circ} \mathrm{C}$. The protoplasts was resuspended with 4 mL SMM buffer ( $0.5 \mathrm{~mol} \mathrm{~L}^{-1}$ sucrose, $0.02 \mathrm{~mol} \mathrm{~L}^{-1} \mathrm{MgCl}_{2}, 0.02 \mathrm{~mol} \mathrm{~L}^{-1}$ maleic acid, pH 6.5 ). The 2 mL prepared protoplast was used to count protoplast formation and regeneration rates. The frequency of protoplast formation and regeneration were measured as reported (Chen et al., 1986). Strains H2011 strain was cultured in TSB broth and its protoplast was prepared.

Protoplast fusion and regeneration: The parental protoplasts in SMM buffer ( $0.5 \mathrm{~mol} \mathrm{~L} \mathrm{~L}^{-1}$ sucrose, $0.02 \mathrm{~mol} \mathrm{~L}{ }^{-1} \mathrm{MgCl}_{2}, 0.02 \mathrm{~mol} \mathrm{~L} \mathrm{~L}^{-1}$ maleic acid, pH 6.5 ) were spined sown and resuspended by SMMD buffer (SMM buffer plus DNase I to a concentration of $5 \mathrm{U} \mathrm{mL}^{-1}$ ). Mixed 0.1 mL protoplasts with 1.8 mL protoplast fusion medium ( $40 \%$, w/v PEG 4000) and incubated in water bath at $37^{\circ} \mathrm{C}$ for 90 sec for protoplast
fusion. Subsequently, 6 mL SCM buffer $\left(0.5 \mathrm{~mol} \mathrm{~L}^{-1}\right.$ sucrose, $0.02 \mathrm{~mol} \mathrm{~L}{ }^{-1} \mathrm{CaCl}_{2}, 0.02 \mathrm{~mol} \mathrm{~L}{ }^{-1}$ maleic acid, pH 6.5 ) was added in and mixed before centrifuging at $3500 \times \mathrm{g}$ for 20 min at $4^{\circ} \mathrm{C}$. The precipitate was resuspended by SMM buffer ( $0.5 \mathrm{~mol} \mathrm{~L}{ }^{-1}$ sucrose, $0.02 \mathrm{~mol} \mathrm{~L}^{-1}$ $\mathrm{MgCl}_{2}, 0.02 \mathrm{~mol} \mathrm{~L}{ }^{-1}$, maleic acid, pH6.5). Then, 0.1 mL samples was primed into the hypertonic selective plate (15 g L ${ }^{-1}$ tryptone, $5 \mathrm{~g} \mathrm{~L}^{-1}$ soytone, $5 \mathrm{~g} \mathrm{~L}^{-1} \mathrm{NaCl}$, $0.5 \mathrm{~mol} \mathrm{~L}^{-1}$ sucrose, $0.02 \mathrm{~mol} \mathrm{~L}^{-1} \mathrm{MgCl}_{2}, 0.02 \mathrm{~mol} \mathrm{~L}^{-1}$ maleic acid, $1.5 \%$ polyvinyl pyrrolidone, $1.5 \%$ agar, $4 \mu \mathrm{~g} \mathrm{~mL}{ }^{-1}$ Rifampicin and $5 \mu \mathrm{~g} \mathrm{~mL}$ - amikacin sulfate) and cultured at $37^{\circ} \mathrm{C}$ for $48 \sim 72 \mathrm{~h}$ until colonies of protoplast fusion regenerated.

Identification and stability test of fusion strains: Colonies on the selective medium were subcultured in a TSA agar plate ( $15 \mathrm{~g} \mathrm{~L}^{-1}$ Tryptone, $5 \mathrm{~g} \mathrm{~L}^{-1}$ Soytone, $5 \mathrm{~g} \mathrm{~L}^{-1} \mathrm{NaCl}$ ) containing $4 \mu \mathrm{~g} \mathrm{~mL}^{-1}$ Rifampicin and $5 \mu \mathrm{~g} \mathrm{~mL}{ }^{-1}$ amikacin sulfate and passed for 15 generations. Identification and stability test of fusion strains was demonstrated through colony morphology, cell-staining characteristics, cultural characteristics, biochemical characteristics, Direct Agglutination test and SDS-PAGE experiments of outer membrane protein.

## RESULTS AND DISCUSSION

Protoplast formation and regeneration: Protoplasts of the parental strains were successfully obtained and regenerated. According to the Chen et al. (1986), the rates of generation and regeneration of protoplast were calculated and listed in Table 1. It showed that the optimized condition for Hps H 2011 is $0.1 \mathrm{~g} \mathrm{~L}^{-1}$

Table 1: Frequencies of protoplast formation and regeneration achieved in the different conditions
Strain

| Concentration of lysozyme ( $\mathrm{mg} \mathrm{mL}^{-1}$ ) | Action time (min) | Protoplast formation frequency (\%) | Regeneration frequency (\%) | Protoplast formation frequency (\%) | Regeneration frequency (\%) |
| :---: | :---: | :---: | :---: | :---: | :---: |
| 10 | 0.05 | 9.78 | 1.92 | 7.41 | 1.83 |
| 10 | 0.10 | 25.87 | 3.97 | 23.68 | 3.43 |
| 10 | 0.20 | 55.81 | 8.07 | 48.76 | 5.37 |
| 10 | 0.40 | 86.76 | 23.09 | 77.56 | 15.36 |
| 15 | 0.05 | 12.25 | 2.84 | 10.32 | 2.78 |
| 15 | 0.10 | 45.68 | 15.36 | 41.67 | 13.63 |
| 15 | 0.20 | 73.78 | 25.83 | 69.58 | 20.61 |
| 15 | 0.40 | 91.87 | 16.52 | 86.67 | 10.32 |
| 20 | 0.05 | 22.63 | 8.87 | 20.06 | 8.39 |
| $\underline{20}$ | 0.10 | 58.57 | 22.81 | 72.53 | $\underline{27.82}$ |
| $\underline{20}$ | 0.20 | $\underline{90.83}$ | 37.32 | 87.13 | 22.43 |
| 20 | 0.40 | 92.69 | 10.73 | 90.89 | 8.26 |
| 25 | 0.05 | 29.53 | 13.24 | 27.34 | 12.42 |
| 25 | 0.10 | 73.33 | 28.38 | 79.36 | 25.71 |
| 25 | 0.20 | 93.62 | 20.45 | 90.06 | 10.85 |
| 25 | 0.40 | 94.09 | 8.79 | 92.07 | 5.73 |
| 30 | 0.05 | 32.46 | 23.77 | 29.07 | 20.67 |
| 30 | 0.10 | 80.93 | 32.09 | 83.03 | 22.64 |
| 30 | 0.20 | 95.76 | 17.73 | 95.76 | 17.73 |
| 30 | 0.40 | 96.42 | 4.76 | 96.42 | 4.76 |

The best conditions and data are shown in boldface type and are underlined
lysozyme and incubation for 20 min . But for sw $E$. coli E2010 the optimized condition is $0.2 \mathrm{~g} \mathrm{~L}^{-1}$ lysozyme for 20 min . The generation rates of protoplasts increased when lysozyme concentration raised in a specific range. However, the regeneration rates would decrease when there was higher lysozyme concentration or longer incubation time.

Regeneration and screening of the fusion strains: The Fusion protoplast of sw E. coli E2010 and Hps H2011were constructed. The hybrid colonies grew well in the hypertonic elective plate and showed resistance characteristics of the parents strains (Fig. 1).

Morphology, cell staining, cultural and biochemical characteristics: The fusion colonies cultured at $37^{\circ} \mathrm{C}$ for 28 h showed similar morphology with sw E. coli E2010 and Hps (Fig. 2). With Gram staining the morphology of fusion strains was Gram-negative bacillus, slightly smaller than sw E. coli E2010 (Fig. 3). The biochemical characteristics of the fusion strains was as same as of Hps H2011 (Table 2). The genetic characteristics of the fusion strains were stable within 15 generations.

Direct agglutination and SDS-PAGE experiment: The direct agglutination test demonstrated that the fusion


Fig. 1: The colony morphology of the fusion strains trained at 52 h in the hypertonic selective medium (containing $4 \mu \mathrm{~g} \mathrm{~mL}{ }^{-1}$ Rifampicin and $5 \mu \mathrm{~g} \mathrm{~mL}{ }^{-1}$ amikacin sulfate)
strains can react with both positive E. coli 2011 serum and Hps serum. By SDS-PAGE test the fusion colonies showed different types of outer membrane protein from its parental strains (Fig. 4).
E. coli and Hps are both Gram-negative bacteria and their cell wall is thick so it is difficult to prepared their


Fig. 2: a) The colony morphology of Escherichia coli E2010 trained at 28 h in the TSA agar plate; b) The colony morphology of the fusion strains trained at 28 h in the TSA agar plate; c) The colony morphology of Hemophilus parasuis H2011 trained at 28 h in the TSA agar plate


Fig. 3: a) The cell morphology (100x) of Escherichia coli E2010 in Gram's solution; b) The cell morphology (x100) of the fusion strains in Gram's stain; c) The cell morphology (x100) of Hemophilus parasuis H2011 in Gram's stain

Table 2: The biochemical characteristics of the fusion strains and the parental strains

| Strains | Items |  |  |  |  |  |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  | Xylose | Maltose | Sucrose | Glucose | MR | Mannitol | $\mathrm{H}_{2} \mathrm{~S}$ | Gelatin | Indole |
| Escherichia coli CH 2012 | + | + | + | + | - | + | - | - | + |
| Hemophilus parasuis FS2011 | - | + | + | + | + | - | - | - | + |
| The fusion strains | - | + | + | + | + | - | - | - | + |

[^1]

Fig. 4: The SDS-PAGE of outer membrane protein of the fusion strains and the parents strains; M: Protein marker; Lane 1: Escherichia coli E2010; Lane 2: Hemophilus parasuis H2011; Lane 3: The fusion strains EH1011
protoplasts. According to previous studies (Weiss, 1976), the optimizing conditions of fusion of sw E. coli E2010 and Hps H 2011 were measured for formation and regeneration of protoplasts.

The protoplasts of sw E. coli E2010 and Hps H2011 were prepared successfully and the fusion protoplasts, EH1011 are constructed for the first time in the world. There are researches on protoplast fusion between intraspecific or intergeneric (Chen et al., 2007; Wang et al., 2009; Ferenczy et al., 1974; Schaeffer et al., 1976; Hopwood et al., 1977). However, intergeneric protoplast fusion of sw E. coli and Hps have not been reported. The fusion strains process their parental antigenicity and are stable in many characteristics and may be used as a bivalent vaccine candidate to control the diseases caused by swine Escherichia coli and Hemophilus parasuis. But as an attenuated live vaccine, the efficacy and safety of the fusion strains needs further study.

## CONCLUSION

There is no reports that protoplast fusion was used to prevent and treat pig diseases. This is the first construction of the protoplast fusion cells of swine Escherichia coli and Hemophilus parasuis which will lay a foundation for the development of a new type of swine bivalent attenuated vaccine to control swine Escherichia coli and Hemophilus parasuis.

## REFERENCES

Bradshaw, R.E., 2006. From protoplasts to gene clusters. Mycologist, 20: 133-139.
Chen, K.A., E.D. Laywell, G. Marshall, N. Walton, T. Zheng and D.A. Steindler, 2006. Fusion of neural stem cells in culture. Exp. Neurol., 198: 129-135.
Chen, W., K. Ohmiya and S. Shimizu, 1986. Protoplast formation and regeneration of dehydrodivanillindegrading strains of Fusobacterium varium and Enterococcus faecium. Applied Environ. Microbial., 52: 612-616.
Chen, Z., J. Wen, Y. Song, Y. Wen and J. Li, 2007. Enhancement and selective production of avermectin B by recombinants of Streptomyces avermitilis via intraspecific protoplast fusion. Chin. Sci. Bull., 52: 616-622.
Ferenczy, L., F. Kevei and J. Zsolt, 1974. Fusion of fungal protoplasts. Nature, 248: 793-794.
Hopwood, D.A., H.M. Wright, M.J. Bibb and S.N. Cohen, 1977. Genetic recombination through protoplast fusion in Streptomyces. Nature, 268: 171-173.
Kielstein, P. and V.J. Rapp-Gabrielson, 1992. Designation of 15 serovars of Haemophilus parasuis on the basis of immunodiffusion using heat-stable antigen extracts. J. Clin. Microbiol., 30: 862-865.
Rafiee, M. and P.J. Blackall, 2000. Establishment, validation and use of the Kielstein-Rapp-Gabrielson serotyping scheme for Haemophilus parasuis. Aust. Vet. J., 78: 172-174.
Schaeffer, P., B. Cami and R.D. Hotchkiss, 1976. Fusion of bacterial protoplasts. Proc. Natl. Acad. Sci., 73: 2151-2155.
Vahle, J.L., J.S. Haynes and J.J. Andrews, 1995. Experimental reproduction of Haemophilus parasuis infection in swine: clinical, bacteriologic and morphologic findings. J. Vet. Diagn. Invest., 7: 476-480.
Wang, C., X. Zhang, Z. Chen, Y. Wen and Y. Song, 2009. Strain construction for enhanced production of spinosad via intergeneric protoplast fusion. Can. J. Microbiol., 55: 1070-1075.
Weiss, R.L., 1976. Protoplast formation in Escherichia coli. J. Bacteriol., 128: 668-670.


[^0]:    Corresponding Author: He Dongsheng, Ministry of Agriculture, Key Laboratory of Biotechnology and Bioproducts Development for Animal Epidemic Prevention, South China Agricultural University, 510642 Guangdong, China

[^1]:    ' + ' On behalf of the test result is positive; '-' represents the test result is negative

