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

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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 O_{107} with resistance to amikacin sulfate and *Hemophilus parasuis* H2011 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,

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stocked in the TSB (17 g L^{-1} tryptone, 3 g L^{-1} multivalent peptone 3 g, 5 g L^{-1} NaCl, 2.5 g L^{-1} K_2HPO_4, 2.5 g L^{-1} glucose) medium was cultured at 37°C for 5 h on a shaker in the TSB medium. The 4 mL well-grown cells were precipitated by 4,000×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 mol L^{-1} Tris, 0.5 mol L^{-1} sucrose, 0.01 mol L^{-1} $CaCl_2$, pH7.0) and preheated lysozyme was added in at series final concentration as 0.05, 0.1, 0.2, 0.4 mg mL^{-1}. The cell suspension mixed with lysozyme were incubated in 37°C water bath for 10, 15, 20, 25 or 30 min, respectively. Then, potassium Ethylene Diamine Tetraacetate (EDTA, 0.1 mol L^{-1}, pH8.0) was added slowly to the mixture at final concentration of 0.01 M and was placed in a 37°C water bath for 15 min. Centrifuged at 3500×g for 20 min at 4°C. The protoplasts was resuspended with 4 mL SMM buffer (0.5 mol L^{-1} sucrose, 0.02 mol L^{-1} $MgCl_2$, 0.02 mol L^{-1} maleic acid, pH6.5). 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 mol L^{-1} sucrose, 0.02 mol L^{-1} $MgCl_2$, 0.02 mol L^{-1} maleic acid, pH6.5) were spined sown and resuspended by SMMD buffer (SMM buffer plus DNase I to a concentration of 5 U 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°C for 90 sec for protoplast fusion. Subsequently, 6 mL SCM buffer (0.5 mol L^{-1} sucrose, 0.02 mol L^{-1} $CaCl_2$, 0.02 mol L^{-1} maleic acid, pH6.5) was added in and mixed before centrifuging at 3500×g for 20 min at 4°C. The precipitate was resuspended by SMM buffer (0.5 mol L^{-1} sucrose, 0.02 mol L^{-1} $MgCl_2$, 0.02 mol L^{-1}, maleic acid, pH6.5). Then, 0.1 mL samples was primed into the hypertonic selective plate (15 g L^{-1} tryptone, 5 g L^{-1} soytone, 5 g L^{-1} NaCl, 0.5 mol L^{-1} sucrose, 0.02 mol L^{-1} $MgCl_2$, 0.02 mol L^{-1} maleic acid, 1.5% polyvinyl pyrrolidone, 1.5% agar, 4 μg mL^{-1} Rifampicin and 5 μg mL^{-1} amikacin sulfate) and cultured at 37°C for 48~72 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 g L^{-1} Tryptone, 5 g L^{-1} Soytone, 5 g L^{-1} NaCl) containing 4 μg mL^{-1} Rifampicin and 5 μg 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 H2011 is 0.1 g L^{-1}

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

		Strain			
		Escherichia coli CH2012		*Hemophilus parasuis* FS2011	
Concentration of lysozyme (mg 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
20	**0.10**	58.57	22.81	**72.53**	**27.82**
20	**0.20**	**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 g 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°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 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

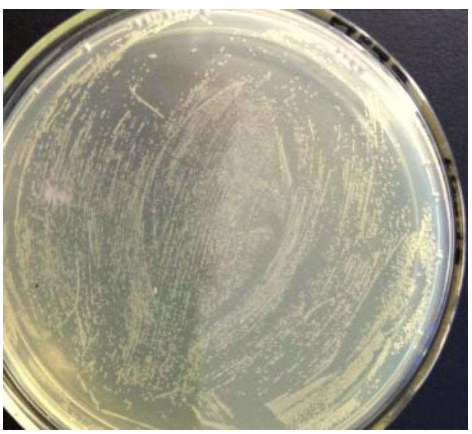

Fig. 1: The colony morphology of the fusion strains trained at 52 h in the hypertonic selective medium (containing 4 μg mL^{-1} Rifampicin and 5 μg mL^{-1} amikacin sulfate)

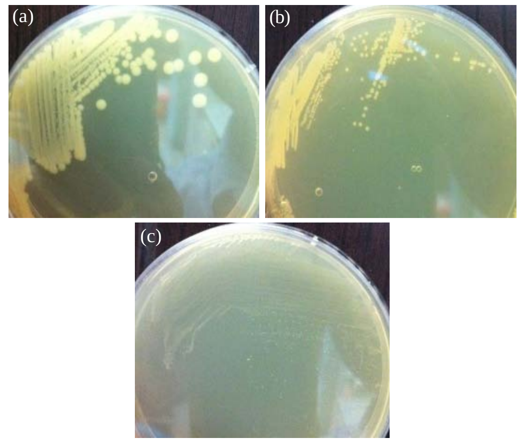

Fig. 2: a) The colony morphology of *Escherichia coli* E2010 trained at 28 h in the TSA agar plate; b) The colony morphology of the fusion strains trained at 28 h in the TSA agar plate; c) The colony morphology of *Hemophilus parasuis* H2011 trained at 28 h in the TSA agar plate

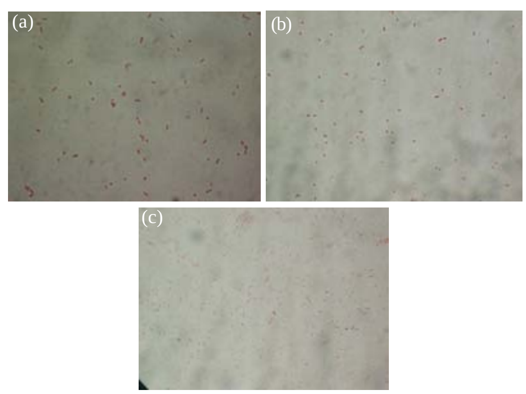

Fig. 3: a) The cell morphology (100x) of *Escherichia coli* E2010 in Gram's solution; b) The cell morphology (x100) of the fusion strains in Gram's stain; c) The cell morphology (x100) of *Hemophilus parasuis* H2011 in Gram's stain

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

	Items								
Strains	Xylose	Maltose	Sucrose	Glucose	MR	Mannitol	H_2S	Gelatin	Indole
Escherichia coli CH2012	+	+	+	+	-	+	-	-	+
Hemophilus parasuis FS2011	-	+	+	+	+	-	-	-	+
The fusion strains	-	+	+	+	+	-	-	-	+

'+' On behalf of the test result is positive; '-' represents the test result is negative

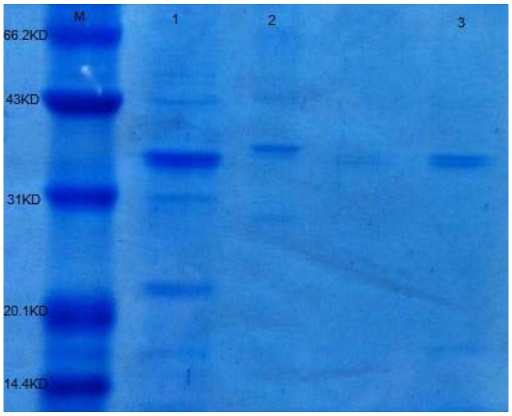

Fig. 4: The SDS-PAGE of outer membrane protein of the fusion strains and the parents strains; M: Protein marker; Lane 1: *Escherichia coli* E2010; Lane 2: *Hemophilus parasuis* H2011; Lane 3: The fusion strains EH1011

protoplasts. According to previous studies (Weiss, 1976), the optimizing conditions of fusion of sw *E. coli* E2010 and Hps H2011 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*.

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