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Impacts of human lysozyme transgene on the microflora of pig feces and the surrounding soil

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ABSTRACT

The rapid development of genetic engineering and extensive applications of genetically engineered (GE) animals have provided many research benefits, but concerns have been raised over the potential environmental impact of transgenic animals. We investigated the effects of human lysozyme (hLZ) transgenic pigs which can express hLZ in their mammary glands on the surrounding environment from the angle of the changes of pig feces and the surrounding soil, including the probability of horizontal gene transfer (HGT), the impact on microbial communities in pig gastrointestinal (GI) tracts and soil, and the influence on the total nitrogen (TN) and total phosphorus (TP) content of pig excrement and surrounding soil. Results showed that hLZ gene was not detected by polymerase chain reaction (PCR) or quantitative real-time PCR (Q-PCR) in gut microbial DNA extracts of manure or microbial DNA extracts of topsoil. PCR-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) analysis and 16S rDNA sequence analysis showed that hLZ gene had no impact on the microflora structure of pig guts or soil. Finally, TN and TP contents were not significantly different in pig manure or soils taken at different distances from the pig site ($P > 0.25$).
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1. Introduction

Animal biotechnology has a long history, which began with the domestication and artificial selection of animals. Modern genetically-based biotechnology only started in the 1960s, following the discovery of the genetic code. However, modern biotechnology can provide new solutions for many problems associated with animal production and health, and offers great opportunities for enhancing productivity and food security (Viljoen, 2005). The first transgenic mice were successfully produced in the early 1980s (Gordon and Ruddle, 1981) and subsequent research on transgenic animals, including fish, rabbits, sheep, pigs, and cows (Cabot et al., 2001; Chan et al., 1998; Du et al., 1992; Hammer et al., 1985; Ozato et al., 1986), has evolved into a major scientific field. From a prospective view, transgene functionality can be divided into four broad categories: environmental adaptability (such as increasing freeze tolerance and increasing disease resistance); improving agricultural performance; producing new or novel products; and producing medications (NRC, 2002).

Despite these benefits, the wide application of transgenic technologies in animals has raised profound concerns in the scientific community and the public. The issues associated with transgenic animals pertain to environmental impact, human food safety, animal health and welfare, trade, and ethics. Environmental issues are considered to be the greatest concern by the scientific community, largely because of the difficulty in identifying environmental problems in the early stage and the difficulty of remediation once a problem has been identified (NRC, 2002). Thus, it is highly important to prioritize the environmental impacts posed by transgenic animals, including impacts verified in the scientific literature and theoretical impacts that have not yet been observed, but these need not be limited to currently developed biotechnology (NRC, 2002).

Animal waste is an important vector whereby animals might exert an influence on the environment. Fecal bacteria and excessive nitrogen and phosphorus are among the most common pollutants affecting rivers and streams (Burkholder et al., 2007). So analysis of feces is very important in the process of environment assessment. Since expression of foreign genes may affect the indigenous microbial ecosystem, detection of the changes of bacteria and nitrogen and phosphorus contents of feces from GE animals is especially crucial.

Horizontal gene transfer (HGT) occurs among different bacterial species (Mathur and Singh, 2005; Quintiliani and Courvalin, 1994), between plants and bacteria (Gasson, 2000; Kay et al., 2002; van den Eede et al., 2004), and between animals and plants (Droge et al., 1998). Traditionally, HGT is considered unlikely to occur from GE animals to bacteria after GE animals are introduced into the

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Table 1
Detailed information of hLZ pigs sampled.

No[a]	Target gene	Promoter	Copy number	Generation	Date of birth	Pig breed	Gene manipulation[b]
1	hLZ	β-casein	1	F0	2007.7	Xiang pig	SCNT
2	hLZ	β-casein	1	F0	2007.7	Xiang pig	SCNT
3	hLZ	β-casein	1	F1	2009.6	Xiang pig	–
4	hLZ	β-casein	1	F1	2009.6	Xiang pig	–
5	–	–	–	–	2007.4	Xiang pig	–
6	–	–	–	–	2007.4	Xiang pig	–
7–8	–	–	–	–	2009.6	Xiang pig	–
9	–	–	–	–	2009.6	Xiang pig	–
10	–	–	–	–	2009.6	Xiang pig	–

[a] 1–4: transgenic positive pigs, 5–6, 9–10: negative control pigs produced by non-transgenic pigs. 7–8: negative control pigs produced by cross breeding between hLZ transgenic-positive F0 pig and a non-transgenic boar, they were full siblings of hLZ transgenic-positive F1 pigs (3 or 4).
[b] SCNT: somatic cell nuclear transformation.

environment, but there are major concerns about the probability of such events and the society need actual and effective evidence with regard to this issue. HGT events can occur in many diverse environments, especially the gastrointestinal (GI) tract (Farthing, 2004). Diverse species of bacteria colonize the GI tract and they may develop natural competence, or the ability to absorb naked DNA (Kelly et al., 2009) like Acinetobacter sp. BD413 (Nielsen et al., 2000, 1997). Bacteria in the GI tract can be analyzed through fecal sampling (Zoetendal et al., 1998). Soil also contains various types of bacteria that may be affected by feces or epidermal cells from GE animals. Thus, the soil microflora can also be used to analyze the probability of HGT from GE animals.

The microbial ecology of GI tract ecosystems is complex and closely interconnected with body metabolism (Hooper et al., 2001; Martin et al., 2008). Integration of a transgene in an endogenous gene and the possible loss of host gene function and inappropriate transgene expression, and exposure of the host to biologically active transgene-derived novel proteins (Buehr et al., 2003; Van Reenen et al., 2001) may lead to metabolic changes in transgenic animals, which may in turn exert an influence on gut flora (Tang et al., 2011). The major functions of the gut microflora include metabolic activities that result in acquisition of energy and absorbable nutrients, important trophic effects on intestinal epithelia and immune structure and function, and protection of the colonized host against invasion by alien microbes (Guarner and Malagelada, 2003; Zoetendal et al., 2008). Changes of the GI tract microflora in the feces might influence the surrounding environments. In addition, metabolic changes and changes of the GI tract microflora may affect the digestion and absorption of nitrogen and phosphorus from the basal diet (Metzler and Mosenthin, 2008), resulting in excessive phosphorus and nitrogen in animal excrement, which could be unfavorable to the environment (Gu et al., 2008). Changes of GI tract microflora or the nitrogen and phosphorus content of animal feces might then influence the surrounding soil. Therefore, investigations aimed at examining changes in microflora and the nitrogen and phosphorus content of transgenic animal excrement and soil will be beneficial to the evaluation of the environmental impacts of transgenic animals.

We generated transgenic pigs expressing recombinant human lysozyme in their mammary glands by somatic cell nuclear transfer, which can benefit the piglets by enhancing their immune function and defend them against pathogenic bacteria. The transgenic pigs expressed recombinant human lysozyme at a level of 0.32 ± 0.01 μg/ml in milk, which was 50-fold higher than pig lysozyme expressed in wild type pigs (Chandan et al., 1968; Tong et al., 2010). The pigs were previously characterized using Southern and Western blots (Tong et al., 2010). We selected this test case and compared their effects on the surrounding environment with wild type pigs over the course of four seasons.

2. Materials and methods

2.1. hLZ expression vector pBC-hLZ

The expression vector pBC-hLZ has been described in detail in previous papers (Tong et al., 2010; Yu et al., 2006). pBC-hLZ expression vector is composed of a promotor, termination region, exon and intron of β-casein, and the hLZ encoding region. The target gene hLZ cDNA was placed under the control of the β-casein promoter, and the 3′ genome DNA of the β-casein was placed downstream of the hLZ cDNA.

2.2. Animals

Two generations of hLZ pigs were used in this study (Table 1). There were two F0 hLZ pigs and two F1 hLZ pigs (1 and 2, 3 and 4 in Table 1). Two pigs were selected for manure sampling from each generation of pigs. Age-matched wild type pigs were selected as controls. All pigs were raised in the same nurturing environment and fed with the same forage and concentrate supplement throughout the year.

2.3. Manure and soil sampling

Ten manure samples were collected (Table 1) and grouped into five manure sets for molecular-based analysis (Table 2). Fresh equal amounts of manure (~100 g) from the same genotyped pigs were thoroughly mixed and transferred into sterile 2 ml and 5 ml centrifuge tubes and kept at −70 °C prior to use. Manure samples (~300 g) were collected from each animal and subjected to nitrogen and phosphorus analysis, after storage at −20 °C. Seasonal groups of manure samples were obtained in October 2009 (autumn), January 2010 (winter), April 2010 (spring), and July 2010 (summer). However, M4 in Table 2 was only collected in October 2009, because of the death of two GE-negative pigs (7 and 8 in Table 1) in November 2009. Attention was paid to avoid contamination during manure sampling by collecting manure of wild type pigs first and changing gloves before each sampling.

Five topsoil samples were collected at distances of 0 m, 15 m, 30 m, 45 m, and 60 m from the pigpen of hLZ transgenic pigs (Table 2), at 0–5 cm depth with each about 200 g for each sample. A mixture of six topsoil samples taken from six random sites equidistant from the pigpen. After thorough mixing, samples were sieved through a 2 mm mesh and transferred to 2 ml and 5 ml sterile centrifuge tubes, and stored at −70 °C prior to use. Topsoil samples (~300 g) subjected to nitrogen and phosphorus analysis were samples collected from the six random sites at each distance point, which were kept at −20 °C before use. Seasonal groups of topsoil samples were collected at the same time as the manure.

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