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In Vitro Development and Transfer of Resistance to Chlortetracycline in *Bacillus subtilis*

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The present criteria and rules controlling the approval of the use of probiotics are limited to antibiotic resistance patterns and the presence of antibiotic resistance genes in bacteria. There is little information available in the literature regarding the risk of the usage of probiotics in the presence of antibiotic pressure. In this study we investigated the development and transfer of antibiotic resistance in *Bacillus subtilis* selected *in vitro* by chlortetracycline in a stepwise manner. *Bacillus subtilis* was exposed to increasing concentrations of chlortetracyclineto induce *in vitro* resistance to chlortetracycline, and the minimal inhibitory concentrations were determinedfor the mutants. Resistant *B. subtilis* were conjugated with *Escherichia coli* NK5449 and *Enterococcus faecalis* JH2-2 using the filter mating. Three *B. subtilis* tetracycline resistant mutants (namely, BS-1, BS-2, and BS-3) were derived *in vitro*. A tetracycline resistant gene, *tet* (K), was found in the plasmids of BS-1 and BS-2. Three conjugates (BS-1N, BS-2N, and BS-3N) were obtained when the resistant *B. subtilis* was conjugated with *E. coli* NK5449. The conjugation frequencies for the BS-1N, BS-2N, and BS-3N conjugates were 4.57×10^{-7}, 1.4×10^{-7}, and 1.3×10^{-8}, respectively. The *tet*(K) gene was found only in the plasmids of BS-1N. These results indicate that long-term use of probiotics under antibiotic selection pressure could cause antibiotic resistance, and the resistance gene could be transferred to other bacteria. The risk arising from the use of probiotics under antibiotic pressure should be considered in the criteria and rules for the safety assessment of probiotics.

Keywords: *Bacillus subtilis*, chlortetracycline, antibiotic resistance, conjugation transfer, *tet*(K), minimal inhibitory concentrations

Introduction

Probiotics are being developed rapidly due to increasing demands of environment-friendly husbandry and human food safety. For example, probiotics are commonly used in piglet feeding to stabilize the gut microflora as a preventive measure during the critical period of weaning (Simon *et al.*, 2007) and in poultry feeding to enhance broiler performance and improve intestinal microbial balance (Mountzouris *et al.*, 2007). Numerous organisms are approved for use in animal feeding by the authorities of the EU (Anadón *et al.*, 2006) and the USA (Pendleton, 2000).

Antibiotics are also a popular way of preventing diseases and improvingproductive performance in animal husbandry around the world. Probiotics are most likely to be exposed to an antibiotic either in feeds or in the gastro-intestinal compartments of animals (Salminen *et al.*, 1998; Siriken *et al.*, 2003), where the antibiotics create a selective pressure on the probiotics for the development of resistance. In fact, antimicrobial resistance in probiotic organisms has been observed (Temmerman *et al.*, 2003; Milazzo *et al.*, 2006; Galopin *et al.*, 2009). In recent years, increased focus has been given to the possibility of resistance transfer from the probiotics to human and animal pathogens, either directly or indirectly, via the commensal flora (Mater *et al.*, 2008; Ouoba *et al.*, 2008). Many antibiotic resistance elements have been found in probiotics that were used as feed additives. Transposon- or plasmid-borne *Tet*(B) was found in the *Bacillus cereus* stain contained in Esporafeed Plus® (SCAN, 1999). The *Bacillus licheniformis* strain in the feed additive AlCare™ was considered unsafe for feeding to pigs because of the risk of transferring resistance to erythromycin (SCAN, 2002).

Bacillus subtilis is one probiotic that is utilized extensively for the therapy and prophylaxis of gastrointestinal (GI) bacterial disorders in humans (Hong *et al.*, 2008; Sorokulova *et al.*, 2008) and as amicrobial feed additive in animals (Anadón *et al.*, 2006; Wannaprasat *et al.*, 2009). In clinical environments it is possible to develop resistance to antibiotics, but very limited information is publicly available about antibiotic resistance development and transfer. Chlortetracycline is one of the most effective antibiotic growth promoters, and it is commonly used in livestock industry in some countries (Jindal *et al.*, 2006; Piddock *et al.*, 2008). There are lots of opportunities for *B. subtilis* to be exposed to chlortetracycline in different environments. *Escherichia coli* and *Enterococci* are the main facultative anaerobic inhabitants of the fecal commensal flora in humans and animals. Commensal bacteria themselves can cause endogenous infections. Moreover, the further threat may be the transfer of their resistance to potentially pathogenic bacteria passing through the gut (Bruinsma *et al.*, 2003). Some investigators have raised concern over the potential of antibiotic-resistance transfer between probiotics and pathogenic bacteria in the gastrointestinal tract (Salyers *et al.*, 2004). The consequences of

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antimicrobial transferable resistance determinants in enteric bacteria that cause infections in human are an increased number of infections and an increased frequency of treatment failures, followed by increased severity of infection (Kruse and Sørum, 1994). For horizontal gene transfer in bacteria, three mechanisms have been identified: natural transformation, conjugation, and transduction. Conjugation is thought to be the main mode of antibiotic resistance gene transfer over species and genus borders (Salyers and Shoemarker, 1995; Davison, 1999). Bacterial mating experiments, including the plate mating and filter mating techniques, can be used to detect whether conjugation has occurred (SCAN, 2003).

At present, most of rules pertaining to the use of probiotics are limited to the determination of antibiotic resistance patterns and the detection of resistant genes before probiotics are permitted for marketing. There is little information available in the literature about the hazard analysis on the risk of probiotics usage under antibiotic pressure. Because probiotics are often exposed to antibiotics in practice, it is necessary to understand whether adaptive resistant mutation will occur during the use of probiotics under long-term antibiotic pressure. It is also necessary to understand whether antibiotic resistance can be transferred to other bacteria, especially pathogens or opportunistic pathogens. In this study, we investigated the emergence and transfer of antibiotic resistance in *B. subtilis* in the presence of chlortetracycline to analyze the hazard of antibiotic resistance caused by the use of probiotics under antibiotic pressure.

Materials and Methods

Drugs and chemicals

Chlortetracycline, tetracycline, doxycycline, gentamicin were supplied by the Institute of Veterinary Drug Control (China). Ciprofloxacin hydrochloride, oxytetracycline and rifampicin were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (China).

Bacteria

Bacillus subtilis ATCC 6633, which is sensitive to most antibiotics (including tetracycline), was obtained from the American Type Culture Collection (USA). *E. coli* NK5449 (CGMCC NO. 1.1437) obtained from the China General Microbiological Culture Collection Center (CGMCC) is rifampicin resistant and was usually used as conjugation recipient. The MIC of rifampicin against *E. coli* NK5449 was ≥200 μg/ml. *E. faecalis* JH2-2 (resistant to rifampicin, MIC>50 μg/ml), obtained from the Belgian Co-ordinated Collections of Micro-organisms (BCCM/LMG), was also used as a recipient in mating experiments.

B. subtilis ATCC 6633 was aerobically cultured at 37°C in nutrient broth (peptone 10 g, beef extract 5 g, sodium chloride 5 g, H_2O 1000 ml, pH 7.4±0.2). *E. coli* NK5449 was cultured at 37°C in nutrient broth. *E. faecalis* JH2-2 was grown in trypticase soy broth (Oxoid, UK). Mueller-Hinton agar (MHA) (Oxoid) with varying concentrations of tetracycline and other tested drugs was used for the determination of MICs.

Determination of MICs

The MICs of antibiotics were tested by the dilution method in Mueller-Hinton agar according to the method described by the Clinical and Laboratory Standards Institute (CLSI, 2008). *E. coli* ATCC 25922 and *E. faecalis* ATCC 29212 were used as quality control organisms. Criteria for identifying Bacillus strains with acquired resistance to tetracycline were applied by SCAN (2003). Consequently, 16 μg/ml was used as the breakpoint concentration, where strains with a tetracycline MIC greater than 16 μg/ml were considered to be resistant mutants.

Selection of resistant *B. subtilis* by chlortetracycline

The selection of resistant mutants was performed according to the multistep method, as described previously (Mushtaq *et al.*, 2004). In brief, 50 μl of *B. subtilis* cultures for which the density reached 10^8 CFU/ml were added to 5 ml fresh nutrient broth with chlortetracycline at one-half the MIC and then cultured overnight at 37°C. Fifty microliters of these cultures were subsequently transferred after overnight incubation to fresh tubes of broth containing either an increasing concentration of antibiotic (MIC, 2MIC, 4MIC, 8MIC, 16MIC, 32MIC) or fresh broth containing no antibiotic (to serve as a positive control). This process was repeated until the bacteria ceased to grow at a given concentration. The MICs of other drugs tested for *B. subtilis* were determined at the beginning and the end of the selection process. To obtain stable mutants, representative clones of resistant strains were subcultured in antibiotic-free nutrient agar plate for 10 serial passages. Then, the MICs of the stable mutants were determined by the CLSI dilution method as described above. Three stable mutants (MIC≧32 μg/ml) were chosen as donors for transfer assay *in vitro*.

Mating experiments

Conjugation of tetracycline resistance was carried out utilizing the method of filter mating (Christie *et al.*, 1987). Conjugation of chlortetracycline-resistant *B. subtilis* and *E. coli* NK5449 was performed on nutrient agar containing 100 μg/ml rifampicin and 32 μg/ml chlortetracycline. A portion of the mating mixture was also serially diluted and spread on chlortetracycline-containing (32 μg/ml) plates. The transfer frequency was the ratio of transconjugants to *B. subtilis* cells.

The transfer of tetracycline resistance between *B. subtilis* and *E. faecalis* JH2-2 was performed according to the procedure described above, with the exception that the selection of transconjugants was performed on brain-heart infusion agar (Oxoid) containing rifampicin (50 μg/ml) and chlortetracycline (32 μg/ml). All mating experiments were repeated three times.

Transconjugants and recipients were confirmed by traditional biochemical assays. The genotypic characterization of the transconjugants was identified by the RiboPrinter® Microbial Characterization System (Qualicon, USA). The stability of the transferred resistance was assessed by transferring transconjugants for 10 serial passages in culture with-

Table 1. Primers for PCR amplification of tetracycline resistant genes in *B. subtilis*

Gene	GenBank No.	Primer name	Primer sequence (5′- 3′)	Product length (bp)	Annealing temperature (°C)
tet(L)	216835	pNS1981(S)	CCGAAATCGGAAGTGTAAT	218	53.0
		pNS1981(A)	GAACGAAAGCCCACCTAA		
tet(L)	532329	tetBSR(S)	CCTGGTAGCAATGGGAGA	119	55.0
		tetBSR(A)	TAAGGAATGGAACGGTGA		
tet(L)	40207	GSY908(S)	CACAGTCAACTTTACGGCACA	314	56.0
		GSY908(A)	TCGGGCTAGAATGAGAATAGGA		
tet(K)	9507372	pNS1(S)	GGGGAATAATAGCACATT	308	49.3
		pNS1(A)	ACCAAGCATAAACGGAAT		

out antibiotics. The MICs of tetracycline and other drugs for the transconjugants were determined by the dilution method described above.

DNA extraction and PCR

The plasmid DNA of *B. subtilis* and the mutants was extracted using the Ultra-pure Mine-Plasmid Rapid Isolation Kit for G^+ (Biodev, China), and the plasmid DNA of *E. coli* and transconjugants was extracted using the B-Type Mini-Plasmid Rapid Isolation Kit (Biodev). The genome DNA of the donors, recipients and transconjugants was extracted using the Axyprep Bacterial Genomic DNA Miniprep Kit (Axygen Biosciences, USA). DNA template (1.0 µl) was added to a PCR mixture containing 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM KCl, each deoxynucleoside triphosphate at a concentration of 200 µM, 0.4 µM each of sense and antisense primer (Sangon, China), and 1 U of Taq DNA polymerase (Dongsheng Biotech, China). Amplifications were carried out by using one thermal cycle consisting of 5 min at 95°C, followed by 35 cycles consisting of 1 min at 94°C, 1 min at different annealing temperature according to different primers (Table 1), and 30 sec at 72°C. The final cycle consisted of 72°C for 10 min. Four primers were designed according to the sequences of two types of tetracycline resistant gene in *B. subtilis*, *tet*(L) and *tet*(K), which were published in the GenBank Database. The sequences of the products were determined by Sangon Biotech Co., Ltd (China).

Results

MICs of resistant strains of *B. subtilis* induced by chlortetracycline

Three typical chlortetracycline resistant *B. subtilis* mutants, namely BS-1, BS-2, and BS-3, were obtained as a result of the selection process. The chlortetracycline MICs for all three *B. subtilis* mutantswere higher than the breakpoint of 16 µg/ml (Table 2 and SCAN, 2003). The MIC of BS-1 increased 4-fold after 21 passages and 16-fold after 34 passages. However, 4-fold increases in the MIC were observed for BS-2 and BS-3 after 24 and 28 passages, respectively. The MICs of tetracycline, oxytetracycline, doxycycline were also higher than the breakpoint, while the MICs of four tetracyclines for the growth control strain BS-0 were much lower than the breakpoint. This observation demonstrates that all three mutants of *B. subtilis* were resistant to four tetracyclines, and chlortetracycline resistant *B. subtilis* had cross-resistance to other tetracyclines. The susceptibility of the mutantsto gentamicin and ciprofloxacin did not change compared to that of the parent strain (shown in Table 2). The MICs for the three resistant *B. subtilis* strains after ten passages on nutrient plates without antibiotics did not change (data not shown), indicating that the chlortetracycline resistance of *B. subtilis* mutants was stable.

Conjugation transfer between resistant *B. subtilis* strains and recipients

Three transconjugants, BS-1N, BS-2N and BS-3N were obtained in all the three conjugation experiments conducted between BS-1, BS-2, BS-3, and *E. coli* NK5449. Phenotypic analysis of the three transconjugants and recipient showed that they were Gram-negative but different from the donors. All of the transconjugants showed the same catabolic profile as the recipient *E. coli* NK5449. They were all lysine decarboxylase, mannitol, glucose, maltose positive and lactose negative. The characteristic of lactose negativity is specific to *E. coli* NK5449 and distinct from other *E. coli* consequently, it can be used to verify the transconjugants without contamination of other *E. coli*. The riboprint patterns from the

Table 2. The MICs of drugs for strains used in this study

Strains	MIC (µg/ml)						
	CTC[a]	TC	OTC	DC	GEN	CIP	RIF
BS-0	8	4	8	4	0.25	0.125	0.031
BS-1	128	64	64	64	0.25	0.125	0.031
BS-2	32	32	32	32	0.25	0.125	0.031
BS-3	32	16	32	32	0.25	0.125	0.031
NK5449	8	4	8	4	0.5	0.031	200
BS-1N	64	32	64	32	0. 5	0.031	200
BS-2N	32	32	16	16	0.5	0.031	200
BS-3N	32	16	16	32	0.5	0.031	200

[a]CTC, chlortetracycline TC, tetracycline OTC, oxytetracycline DC, doxycycline GEN, gentamicin CIP, ciprofloxacin hydrochloride RIF, rifampicin

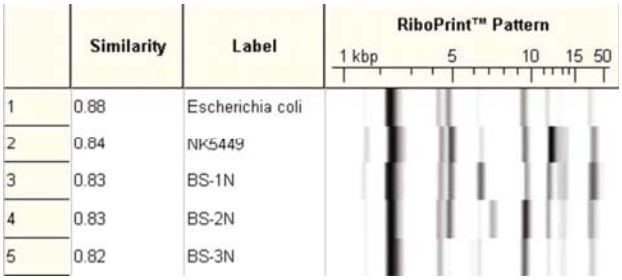

Fig. 1. Three conjugates (BS-1N, BS-2N, and BS-3N) ribopatterns were compared with the patterns from *E. coli* NK5449.

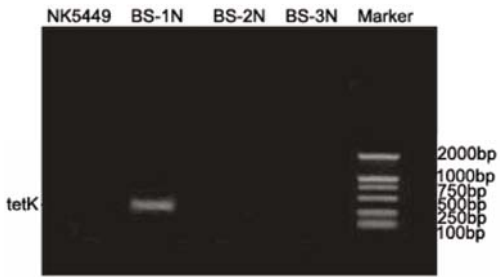

Fig. 3. Gel electrophoresis of PCR products of the plasmids in receipt and transconjugants with primer pNS1.

*Eco*RI enzyme further verified that the three transconjugants were *E. coli* NK5449 with pattern similarity greater than 95% (Fig. 1). The conjugation frequencies of BS-1, BS-2, and BS-3 with *E. coli* NK5449 were 4.57×10^{-7}, 1.4×10^{-7}, and 1.3×10^{-8}, respectively. The MIC values (Table 2) of the tetracyclines showed that all of the transconjugants displayed MICs of $\geqq$6 μg/ml, higher than that of *E. coli* NK5449, indicating that the transconjugants acquired resistance to tetracyclines from the donor. Three transconjugants remained susceptible to gentamicin and ciprofloxacin. After the transconjugants had been serial transferred three times on the nutrient plates lacking antibiotics, the MICs returned to the same level as that of NK5449 (data not shown). This indicates that the chlortetracycline resistance of the transconjugants was not stable in the absence of selective pressure. Transconjugants were not successfully obtained by filter mating between three *B. subtilis* mutants and *E. faecalis* JH2-2 (results not shown).

Identification of chlortetracycline resistant gene in *B. subtilis* mutants and transconjugants

The *tet*(K) gene was amplified in the plasmid DNA template from BS-1, BS-2, and transconjugant BS-1N (Figs. 2 and 3), but it was not amplified in genomic DNA. No DNA could be amplified with primers specific for the *tet*(L) gene (which is mainly responsible for tetracycline resistance in *B. subtilis* mutants) and the total DNA (plasmid DNA and genomic DNA) of all *B. subtilis* strains and transconjugants as a template. This result indicated that BS-1 and BS-2 harbored plasmids carrying the *tet*(K) gene, which conferred chlortetracycline resistance to *B. subtilis*. BS-1N with transferred marker indicated that the *tet*(K) gene could be transferred from *B. subtilis* to *E. coli* by conjugation. The lack of amplification products from BS-3, BS-2N, and BS-3N indicates that these strains may contain some other potential resistance genes other than *tet*(K) and *tet*(L).

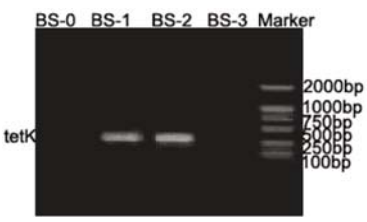

Fig. 2. Gel electrophoresis of PCR products of the plasmids in *B. subtilis* with primer pNS1. BS-0 represents chlortetracycline sensitive *B. subtilis* strain. BS-1, BS-2, and BS-3 represent three resistant mutants.

Discussion

Adaptive mutation phenomena often occur when bacteria are cultured in the presence of a selective agent (Foster, 2007). In this study, we found that resistant *B. subtilis* mutants could be obtained when a sensitive strain was incubated with chlortetracycline. It took approximately 15 passages for three resistant *B. subtilis* mutants to increase the chlortetracycline MIC from 8 μg/ml to 16 μg/ml. This result demonstrated that the development of *B. subtilis* resistance to chlortetracycline requires a long time exposure to the antibiotic. The *tet* efflux genes coding for membrane-associated proteins that export tetracycline from the cell, *Tet*(K) and *Tet*(L), are found primarily in Gram-positive species, such as *B. subtilis* (Chopra and Roberts, 2001). Moreover, the *tet*(L) gene is usually reported to confer tetracycline resistance to *B. subtilis* (Hoshino *et al.*, 1985; Sakaguchi *et al.*, 1988). However, we found that two of three mutants carried the *tet*(K) gene in a plasmid but not the *tet*(L) gene. This finding indicates that the occurrence of resistance to chlortetracycline in *B. subtilis* is caused by *tet*(K) which, which is responsible for the efflux of tetracyclines. In addition, we were unable to observe *tet*(K) or *tet*(L) genes carried on a plasmid or on the chromosome of the third mutant *B. subtilis*. This finding implies that an unknown gene may confer resistance to tetracyclines in *B. subtilis*. This novel mechanism of resistance requires further analysis.

Reports of the conjugative transfer of antibiotic resistance genes between Bacillus species and other bacteria are rare (Trieu-Cuot *et al.*, 1987; Bertram *et al.*, 1991). Trieu-Cuot *et al.* (1987) found that the prototype vector constructed plasmid, pAT187, which contains a kanamycin resistance gene, was successfully transferred to *Enterococcus faecalis*, *Streptococcus lactis*, *Streptococcus agalactiae*, *Bacillus thuringiensis*, *Listeria monocytogenes*, and *Staphylococcus aureus* by filter mating at frequencies of 2×10^{-8} to 5×10^{-7}. An *E. coli* strain carrying Tn916 yielded transconjugants with *Bacillus subtilis*, *Clostridium acetobutylicum*, *Enterococcus faecalis*, and *Streptococcus lactis* subsp. *diacetylactis* by mating at frequencies of 10^{-4} to 10^{-6} (Bertram *et al.*, 1991). In fact, naturally occurring plasmids in *Bacillus*species are common, and many of them encode conjugative or mobile elements (Hong *et al.*, 2005). Approximately 30% of *B. subtilis* strains carry small cryptic plasmids, and all of the small cryptic plasmids found in the soil isolates of *B. subtilis* contain the *mob* gene, which mediates gene transfer, suggesting that the conjugal transfer of

bacillus plasmids is widely spread in nature (Meijer *et al*., 1998; Prozorov, 2003).

E. coli, a type of opportunistic pathogen, is very common in the intestinal tract and environment. Evidence is accumulating to support the hypothesis that intestinal bacteria not only exchange resistance genes among themselves but might also interact with bacteria that are passing through the colon, causing these bacteria to acquire and transmit antibiotic resistance genes (Levy and Marshall, 2004; Salyers *et al*., 2004). If *E. coli* obtains chlortetracycline resistance genes from *B. subtilis* and then transfers these genes to other bacteria passing through the colon, it may cause an accumulation of tetracycline resistant genes in the environment, which represents a great potential threat for the treatment of animal and human diseases. Therefore, when considering all current evidence for the potential donor and recipient, it may be suggested that *B. subtilis* can act as a reservoir of acquired antimicrobial resistance genes that can be transferred to other bacteria.

In the present study, no conjugation between the intraspecies *B. subtilis* and *E. faecalis* occurred under the experimental *in vitro* conditions (data not shown). This result differed from the previous finding, which demonstrated the transfer of tetracycline resistance from *B. subtilis* cells carrying tetracycline-resistance plasmid pCF10 to *S. faecalis* strains in filter matings (Christie *et al*., 1987). It is possible that restriction- modification systems limit gene exchange between the two bacteria, or the mismatch-repair system inhibits interspecies recombination (Matic *et al*., 1996).

Exposure of probiotics to antibiotics often occurs in human and animal intestinal tracts and in the environment. One of the problems with probiotic usage in humans is that probiotics are prescribed as an adjunct to antibiotics in some countries. This is a common practice in Southeast Asia, where probiotic bacteria are used to limit the side effects of antibiotics (Hong *et al*., 2005). In animal feeding practice, probiotics are commonly used with antibiotics as growth promoters to provide additional benefits. This latter usage may be very dangerous–it might lead to the emergence of resistant mutants and the transfer of resistant genes to pathogens or opportunistic pathogens. The emergence of such mutants may pose a great risk to animal health and, ultimately, to human health.

A number of rules and criteria have been created to ensure the safety of probiotics with respect to antibiotic resistance by different international organizations and countries. Guidelines for the Evaluation of Probiotics in Food were issued by FAO/WHO, which noted that the antibiotic resistance patterns of all probiotics should be determined before they are approved for marketing (FAO/WHO, 2002). The criteria for the safety assessment of probiotic antibiotic resistance issued by Europe are one of the strictest sets of rules in this category (SCAN, 2003). According to the European criteria, all bacteria products used as feed additives must be examined to establish the susceptibility of the component strains to a relevant range of antibiotics. This criterion states that the MICs of the probiotics should be lower than the breakpoints, or that the resistance has been proven to be intrinsic and thus would not be transferred to other bacteria. However, the risk of antibiotic resistant gene transfer between probiotics and other intestinal bacterial under long-term exposure to antibiotics has not been considered in all sets ofcriteria and rules until now. In this study, we found that tetracycline-sensitive *B. subtilis* strains could develop resistance when they were incubated with chlortetracycline over time and that their resistance could be transferred to *E. coli.* As stated above, probiotics have a number of opportunities to be exposed to antibiotics in practice. This exposure may lead to the emergence of antibiotic resistance and gene transfer between pathogens or opportunistic pathogens. Considering the prevalence of resistant genes, we suggest that safety assessment regarding the exposure of probiotics to antibiotics for a long period of time should be performed before probiotics are approved. The model used in this study is simple. A more complicated model such as a gnotobiotic animal model could be considered to enhance this assessment (Jacobsen *et al*., 2007).

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References

Anadón, A., Martínez-Larrañaga, M.R., and Aranzazu Martínez, M. 2006. Probiotics for animal nutrition in the European Union. Regulation and safety assessment. *Regul. Toxicol. Pharmacol.* **45**, 91–95.

Bertram, J., Strätz, M., and Dürre, P. 1991. Natural transfer of conjugative transposon Tn916 between Gram-positive and Gram-negative bacteria. *J. Bacteriol.* **173**, 443–448.

Bruinsma, N., Stobberingh, E., de Smet, P., and van den Bogaard, A. 2003. Antibiotic use and the prevalence of antibiotic resistance in bacteria from healthy volunteers in the dutch community. *Infection* **31**, 9–14.

Chopra, I. and Roberts, M. 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol. Mol. Biol. Rev.* **65**, 232–260.

Christie, P.J., Korman, R.Z., Zahler, S.A., Adsit, J.C., and Dunny, G.M. 1987. Two conjugation systems associated with *Streptococcus faecalis* plasmid pCF10: identification of a conjugative transposon that transfers between *S. faecalis* and *Bacillus subtilis*. *J. Bacteriol.* **169**, 2529–2536.

CLSI. 2008. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals (M31-A3), Third Edition.

Davison, J. 1999. Genetic exchange between bacteria in the environment. *Plasmid* **42**, 73–91.

FAO/WHO. 2002. Guidelines for the Evaluation of Probiotics in Food. http://www.who.int/entity/foodsafety/fs_management/en/probiotic_guidelines.pdf

Foster, P.L. 2007. Stress-induced mutagenesis in bacteria. *Crit. Rev. Biochem. Mol. Biol.* **42**, 373–397.

Galopin, S., Cattoir, V., and Leclercq, R. 2009. A chromosomal chloramphenicol acetyltransferase determinant from a probiotic strain of *Bacillus clausii*. *FEMS Microbiol. Lett.* **296**, 185–189.

Hong, H.A., Duc, H., and Cutting, S.M. 2005. The use of bacterial spore formers as probiotics. *FEMS Microbiol. Rev.* **29**, 813–835.

Hong, H.A., Huang, J.M., Khaneja, R., Hiep, L.V., Urdaci, M.C., and Cutting, S.M. 2008. The safety of *Bacillus subtilis* and *Bacillus indicus* as food probiotics. *J. Appl. Microbiol.* **105**, 510–520.

Hoshino, T., Ikeda, T., Tomizuka, N., and Furukawa, K. 1985. Nucleotide sequence of the tetracycline resistance gene of pTHT15, a thermophilic Bacillus plasmid: comparison with staphylococcal TcR controls. *Gene* **37**, 131–138.

Jacobsen, L., Wilcks, A., Hammer, K., Huys, G., Gevers, D., and Andersen, S.R. 2007. Horizontal transfer of tet(M) and erm(B) resistance plasmids from food strains of *Lactobacillus plantarum* to *Enterococcus faecalis* JH2-2 in the gastrointestinal tract of gnotobiotic rats. *FEMS Microbiol. Ecol.* **59**, 158–166.

Jindal, A., Kocherginskaya, S., Mehboob, A., Robert, M., Mackie, R.I., Raskin, L., and Zilles, J.L. 2006. Antimicrobial use and resistance in swine waste treatment systems. *Appl. Environ. Microbiol.* **72**, 7813–7820.

Kruse, H. and Sørum, H. 1994. Transfer of multiresistance plasmids between bacteria of diverse origin in natural micro-environments. *Appl. Environ. Microbiol.* **60**, 4015–4021.

Levy, S.B. and Marshall, B. 2004. Antibacterial resistance worldwide: causes, challenges and responses. *Nat. Med.* **10**, S122–129.

Mater, D.D., Langella, P., Corthier, G., and Flores, M.J. 2008. A probiotic *Lactobacillus* strain can acquire vancomycin resistance during digestive transit in mice. *J. Mol. Microbiol. Biotechnol.* **14**, 123–127.

Matic, I., Taddei, F., and Radman, M. 1996. Geneticbarriers among bacteria. *Trends Microbiol.* **4**, 69–72.

Meijer, W.J., Wisman, G.B., Terpstra, P., Thorsted, P.B., Thomas, C.M., Holsappel, S., Venema, G., and Bron, S. 1998. Rolling-circle plasmids from *Bacillus subtilis*: complete nucleotide sequences and analyses of genes of pTA1015, pTA1040, pTA1050 and pTA1060, and comparisons with related plasmids from Gram-positive bacteria. *FEMS Microbiol. Rev.* **21**, 337–368.

Milazzo, I., Speciale, A., Musumeci, R., Fazio, D., and Blandino, G. 2006. Identification and antibiotic susceptibility of bacterial isolates from probiotic products available in Italy. *New Microbiol.* **29**, 281–291.

Mountzouris, K.C., Tsirtsikos, P., Kalamara, E., Nitsch, S., Schatzmayr, G., and Fegeros, K. 2007. Evaluation of the efficacy of a probiotic containing *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, and *Pediococcus* strains in promoting broiler performance and modulating cecal microflora composition and metabolic activities. *Poult. Sci.* **86**, 309–317.

Mushtaq, S., Ge, Y., and Livermore, D.M. 2004. Doripenem versus *Pseudomonas aeruginosa in vitro*: Activity against characterized isolates, mutants, and transconjugants and resistance selection potential. *Antimicrob. Agents Chemother.* **48**, 3086–3092.

Ouoba, L.I., Lei, V., and Jensen, L.B. 2008. Resistanceof potential probiotic lactic acid bacteria and bifidobacteria of African and European origin to antimicrobials: determination and transferability of the resistance genes to other bacteria. *Int. J. Food Microbiol.* **121**, 217–224.

Pendleton, B. 2000. The regulatory environment-direct-fed microbial, enzyme & forage additive compendium. microbialcompendium.com.

Piddock, L.J., Griggs, D., Johnson, M.M., Ricci, V., Elviss, N.C., Williams, L.K., Jørgensen, F., Chisholm, S.A., Lawson, A.J., Swift, C., and *et al.* 2008. Persistence of Campylobacter species, strain types, antibiotic resistance and mechanisms of tetracycline resistance in poultry flocks treated with chlortetracycline. *J. Antimicrob. Chemother.* **62**, 303–315.

Prozorov, A.A. 2003. Conjugation in bacilli. *Microbiology* **72**, 517–527.

Sakaguchi, R., Amano, H., and Shishido, K. 1988. Nucleotide sequence homology of the tetracycline-resistance determinant naturally maintained in *Bacillus subtilis* Marburg 168 chromosome and the tetracycline-resistance gene of *B. subtilis* plasmid pNS1981. *Biochim. Biophys. Acta.* **950**, 441–444.

Salminen, S., von Wright, A., Morelli, L., Marteau, P., Brassart, de Vos, W.M., Fonden, R., Saxelin, M., Collins, K., Mogensen, G., and *et al.* 1998. Demonstration of safety of probiotics. *Int. J. Food Microbiol.* **44**, 93–106.

Salyers, A.A., Gupta, A., and Wang, Y. 2004. Human intestinal bacteria as reservoirs for antibiotic resistance genes. *Trends Microbiol.* **12**, 412–416.

Salyers, A.A. and Shoemaker, N.B. 1995. Conjugative transposons: the force behind the spread of antibiotic resistance genes among *Bacteroides* clinical isolates. *Anaerobe* **1**, 143–150.

SCAN. 1999. Assessment by the scientific committee on animal nutrition (SCAN)(R) of a micro-organisms product: Esporafeed Plus® http://ec.europa.eu/food/fs/sc/scan/ out39_en.pdf.

SCAN. 2002. Opinion of the scientific committee on animal nutrition on the use of *Bacillus licheniformis* NCTC 13123 in feeding stuffs for pigs (Product AlCare™). http://ec.europa.eu/food/fs/sc/scan/out79_en.pdf.

SCAN. 2003. Opinion of the scientific committee on animal nutrition on the criteria for assessing the safety of micro-organisms resistant to antibiotics of human clinical and veterinary importance. http://ec.europa.eu/food/fs/ sc/scan/out108_en.pdf

Simon, O., Vahjen, W., and Taras, D. 2007. Potentials of probiotics in pig nutrition. *Feed Mix.* **15**, 25–27.

Siriken, B., Bayram, I., and Onol, A.G. 2003. Effects of probiotics: alone and in a mixture of Biosacc® plus Zinc Bacitracin on the caecal microflora of Japanese quail. *Res. Vet. Sci.* **75**, 9–14.

Sorokulova, I.B., Pinchuk, I.V., Denayrolles, M., Osipova, I.G., Huang, J.M., Cutting, S.M., and Urdaci, M.C. 2008. The safety of two Bacillus probiotic strains for human use. *Dig. Dis. Sci.* **53**, 954–963.

Temmerman, R., Pot, B., Huys, G., and Swings, J. 2003. Identification and antibiotic susceptibility of bacterial isolates from probiotic products. *Int. J. Food Microbiol.* **81**, 1–10.

Trieu-Cuot, P., Carlier, C., Martin, P., and Courvalin, P. 1987. Plasmid transfer by conjugation from *Escherichia coli* to Gram-positive bacteria. *FEMS Microbiol. Lett.* **48**, 289–294.

Wannaprasat, W., Koowatananukul, C., Ekkapobyotin, C., and Chuanchuen, R. 2009. Quality analysis of commercial probiotic products for food animals. *Southeast Asian J. Trop. Med. Public Health* **40**, 1103–1112.