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Bacillus subtilis Spores Germinate in the Chicken Gastrointestinal Tract▽

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A number of poultry probiotics contain bacterial spores. In this study, orally administered spores of *Bacillus subtilis* germinated in the gastrointestinal (GI) tracts of chicks. Furthermore, 20 h after spores were administered, vegetative cells outnumbered spores throughout the GI tract. This demonstrates that spore-based probiotics may function in this host through metabolically active mechanisms.

Antibiotic feed supplements have been used in commercial poultry farming for over 50 years due to their growth-promoting and prophylactic properties (3, 7, 11). However, their use in the European Union (EU) was banned in 2006 as part of an initiative aimed at promoting the prudent use of antibiotics (1, 18, 24). As a result, there is now a market for alternative growth-promoting and prophylactic products. In general, feed supplements remain the method of choice, due to the scale and economics of modern poultry farming.

Probiotics are one form of alternative feed supplement or "functional food." They can be defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (10). Many probiotic products are already available for commercially farmed poultry, and a number of these contain or consist of bacterial spores, principally of the genus *Bacillus* (4, 5, 16). Bacterial spores are particularly well suited for use as live microbial products as they are metabolically dormant and highly resilient to environmental stresses. These intrinsic properties are highly desirable from a commercial perspective and mean that spore-based products have a long shelf life and retain their viability during distribution and storage. Despite this, knowledge of how bacterial spores may function as probiotics is severely limited at present.

Considering that bacterial spores are metabolically dormant, it is of particular interest to question whether or not they are able to germinate in the chicken gastrointestinal (GI) tract. This is an important point to address, as it is widely accepted that microorganisms must be in a viable state to function as probiotics, the implication being that one or more metabolic processes are essential for probiotic function in the host GI tract. Indeed, if spores do not germinate in the chicken GI tract, it is difficult to envisage how they may function as probiotics through mechanisms which require them to be metabolically active (e.g., secretion of antimicrobial compounds and competition for essential nutrients).

Early work with mammalian hosts suggested that bacterial spores are able to germinate in the GI tracts of dogs, rabbits, and mice (13, 15). Definitive proof of this was obtained when it was shown that vegetative cells of *Bacillus subtilis* could be detected in the small intestines of mice after they had received an oral dose of spores (6, 25). More recently, spore germination has been observed in the GI tracts of pigs, using flow cytometry analysis (17). While all of these findings are important in the context of mammalian hosts, it cannot be assumed that they will translate directly to an avian host, primarily due to differences in GI anatomy and physiology. Consequently, in the wake of the EU ban on antimicrobial feed supplements and in view of the fact that a number of spore-based probiotic products are already available for commercially farmed poultry, in this study we addressed the pertinent question of whether or not spores of the model spore-forming bacterium *B. subtilis* are able to germinate in the chicken GI tract. We used a 1-day-old chick model as this is the approximate age of chickens when they are first housed within broiler sheds and placed on diets supplemented with "functional foods," such as antibiotics or probiotics.

Spores are transient members of the chick GI tract. To address the fate of orally administered spores, 1-day-old specific pathogen-free White Leghorn chicks were each dosed by oral gavage with 1×10^9 spores of *B. subtilis* SC2362 (6, 8, 9) suspended in 0.1 ml of sterile distilled water. This strain of *B. subtilis* harbors a unique *rrnO-lacZ* fusion gene and a chloramphenicol acetyltransferase (*cat*) gene in the chromosome. Spores were prepared as described previously (22), including sequential treatment with lysozyme (20 mg/ml) and heat (68°C for 90 min) to ensure there were no contaminating vegetative cells present. Three chicks were selected at random for sampling at 6, 12, 20, 48, and 168 h after spore doses were administered. Sections of duodenum, jejunum, ileum, cecum, and colon were excised from each chick postmortem, and the number of *B. subtilis* SC2362 spores per gram of tissue (including luminal contents) was determined by heat treatment (to kill vegetative cells and background contaminants), serial dilution, and plating. A mean value was calculated for the number of spores in the duodenum, the jejunum, and the ileum of each chick and reported as the number of spores per gram of small intestine. Numbers of *B. subtilis* SC2362 spores were found to decrease with time, throughout the GI tract, indicating that spores are transient members of the chick GI microflora (Fig. 1). This may have been due to spore germination in the GI tract and/or shedding of spores in feces. Between 5×10^2

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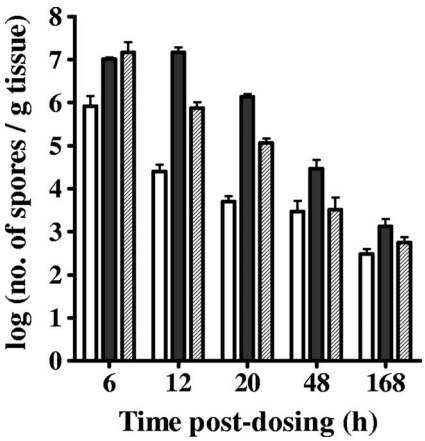

FIG. 1. Enumeration of *B. subtilis* SC2362 spores in chick GI tissues. Tissue samples were taken postmortem at 6, 12, 20, 48, and 168 h after 1×10^9 spores had been administered to each chick. The number of spores in the small intestine (open bars), cecum (solid bars), and colon (cross-hatched bars) of each chick was determined by serial dilution and plating, as described in the text. Bars are mean results for three chicks sampled. Error bars are standard errors of the means. No spore formers were recovered from any tissue excised from three undosed (negative) control chicks (not shown).

and 5×10^3 spores remained in the GI tract of each chick at the end of the experimental period.

Spore germination in the chick GI tract. To investigate the possibility of spore germination in the GI tracts of chicks, a reverse transcriptase PCR (RT-PCR)-based method was developed for detecting vegetative cells of *B. subtilis* SC2362. We decided to use a molecular approach, as preliminary work revealed that the process of serial dilution and plating onto medium supplemented with chloramphenicol was an unreliable method for accurately determining total viable counts of *B. subtilis* SC2362 (i.e., spores and vegetative cells) in samples of gut tissue. Other researchers have reported similar problems (14, 15), the issues being (i) inefficient and nonsynchronous spore germination in the absence of a heat treatment/activation step, which leads to an underestimation of total viable units, and (ii) growth of background contaminants which obscure colonies of *B. subtilis* on agar plates. Ultimately, our decision to use RT-PCR was based on successful work by researchers elsewhere (6, 25).

In the first instance, a semiquantitative RT-PCR assay was developed for detecting vegetative cells of *B. subtilis* SC2362. Total RNA was prepared from 30-mg sections of gut tissue (including luminal contents), using an RNeasy mini-kit (Qiagen), and RNA concentrations were determined by spectroscopy prior to RT-PCR. The primers SC2362-F (GGGAGGGTCA TTGGAAACTG) and SC2362-R (CCTATATAAAAGCATT AGTGTATCAACAAGC) were designed using Primer Express software (Applied Biosystems) to target the unique *rrnO-lacZ* sequence of *B. subtilis* SC2362. The assay was then validated using sections of control tissue spiked with known numbers of vegetative cells. A visible RT-PCR product of the expected size (117 bp) was amplified when total RNA from gut tissue spiked with $\geq 1 \times 10^4$ vegetative cells of *B. subtilis* SC2362 was used as the template (Fig. 2). The absolute detection limit of the assay was found to be 100 cells or 3.33×10^5 cells per gram of tissue. (Total RNA was extracted from sections of tissue that weighed 30 mg and were eluted in 50 μl. A 0.5-μl volume of eluate was then used as the template in RT-PCR). No RT-PCR product was amplified from RNA extracted from the parental *B. subtilis* strain PY79 (26) or from spores of *B. subtilis* SC2362, indicating that the assay was specific for the *rrnO-lacZ* mRNA of *B. subtilis* SC2362 vegetative cells (data not shown). Additional RT-PCRs with an initial RT denaturing step (95°C for 30 min) or with primers Bact-F (CTGGTCGTACCACTGGTATTG) and Bact-R (CAGGTG GGGCAATGATCTTG), used to amplify a 550-bp product from chicken β-actin mRNA (2), confirmed that RT-PCR products were not amplified from contaminating DNA and that the RNA preparation procedure was consistent between samples (data not shown).

To determine whether or not orally administered spores of *B. subtilis* SC2362 germinated in the GI tracts of chicks, RT-PCR was carried out using RNA template from sections of gut tissue which had been sampled from chicks at 6, 12, 20, 48, and 168 h after they had been dosed with spores. The *rrnO-lacZ* RT-PCR product was amplified from RNA template prepared from the ceca and colons of two of the three chicks sampled at 12 h and all three of the chicks sampled 20 h after spore doses were administered (Fig. 3). Repeat reactions with an initial RT

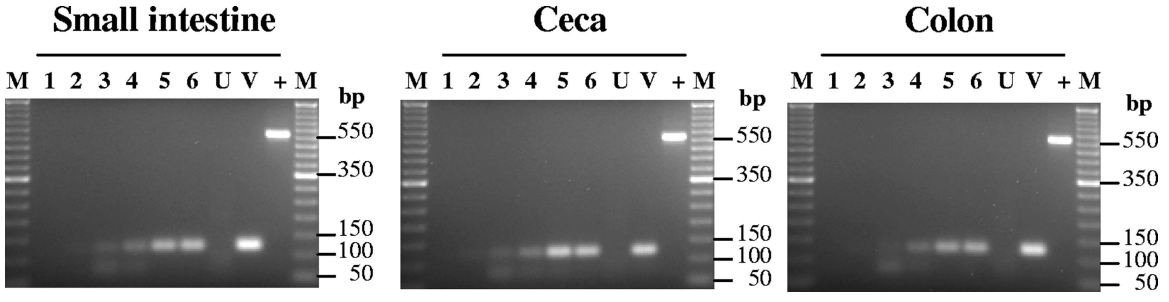

FIG. 2. Validation and sensitivity of the semiquantitative RT-PCR assay for detecting vegetative cells of *B. subtilis* SC2362. RT-PCR was carried out with the primers SC2362-F and SC2362-R, using template RNA prepared from sections of chick GI tissue (30 mg) spiked with known numbers of vegetative cells. Lanes: 1, 1×10^3 vegetative cells; 2, 5×10^3 vegetative cells; 3, 1×10^4 vegetative cells; 4, 5×10^4 vegetative cells; 5, 1×10^5 vegetative cells; 6, 5×10^5 vegetative cells; U, unspiked GI tract tissue control; V, total RNA prepared from 1×10^6 *B. subtilis* SC2362 vegetative cells; +, RT-PCR control reaction supplied by the manufacturer (550-bp product from rabbit globin mRNA); M, 50-bp ladder (Invitrogen).

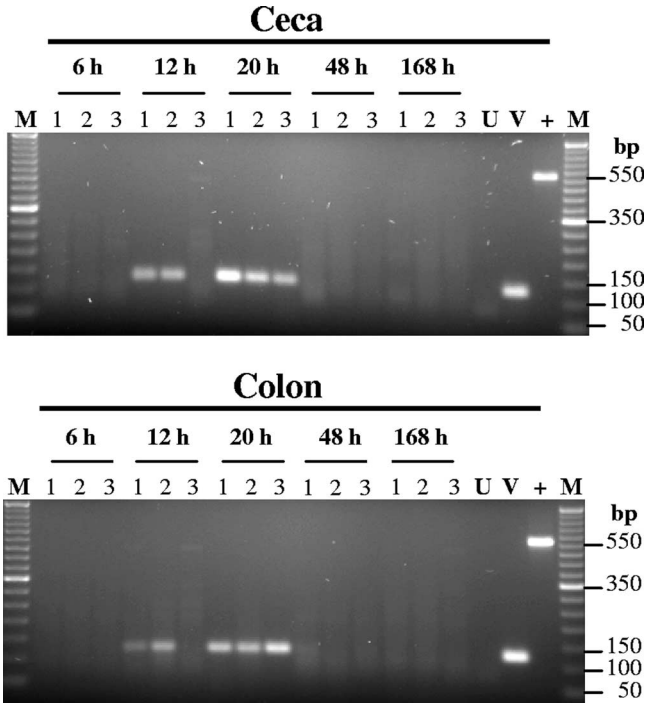

FIG. 3. Detection of *B. subtilis* SC2362 vegetative cells (*rrnO-lacZ*) in chick tissues by semiquantitative RT-PCR. Three chicks (1, 2, and 3) were sampled at 6, 12, 20, 48, and 168 h after 1×10^9 spores had been administered. Total RNA was prepared from sections of GI tract tissue (30 mg) and used as template in RT-PCR with the primers SC2362-F and SC2362-R. A 117-base pair product was generated if $\geq 3.33 \times 10^5$ vegetative cells per gram of tissue was present in the sample. U, pooled RNA template from samples of GI tissue excised from the three undosed (negative) control chicks; V, total RNA prepared from 1×10^6 vegetative cells of *B. subtilis* SC2362; +, RT-PCR control reaction supplied by manufacturer (550-bp product from rabbit globin mRNA); M, 50-bp ladder (Invitrogen).

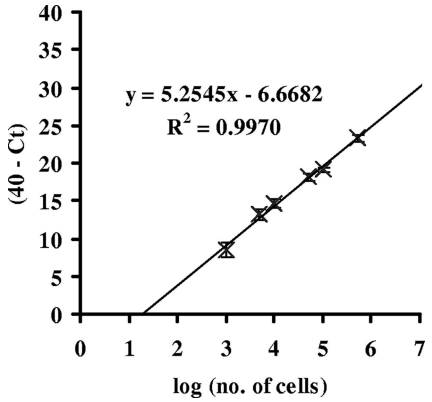

FIG. 4. qRT-PCR calibration curve. The equation of the line describes the relationship between the $\log_{10}$(number of *B. subtilis* SC2362 vegetative cells in a 30-mg section of chick GI tract tissue) and the qRT-PCR signal strength (40 − cycle threshold [*Ct*] value), when a 0.1 volume of total RNA (i.e., 5 μl) was used as the template. Results are mean (40 − *Ct*) values calculated for all tissues spiked with equivalent numbers of *B. subtilis* SC2362 vegetative cells, irrespective of which region of the chick GI tract the tissue was from. No qRT-PCR signal was detected in negative control reactions which contained no template, total RNA from 1×10^9 spores of *B. subtilis* SC2362, total RNA from 1×10^9 vegetative cells of *B. subtilis* PY79 or total RNA from unspiked (negative) control chick tissue (not shown). All standards were analyzed in duplicate on four separate occasions.

denaturing step or with the primers Bact-F and Bact-R confirmed that these were true positives (i.e., not amplified from contaminating DNA) and that the RNA preparation procedure was consistent between all samples (data not shown). As samples from the 12- and 20-h time points were the only ones to test positive for *B. subtilis* SC2362 vegetative cells (indicating the presence of at least 3.33×10^5 cells per gram of tissue), we proceeded to analyze these samples by TaqMan real-time quantitative RT-PCR (qRT-PCR) to estimate the numbers of vegetative cells present.

For qRT-PCR, the primers SC2362-F and SC2362-R were used in conjunction with the fluorescently labeled TaqMan probe, SC2362-P ([FAM]-TTCCACTCTCCTCTTCTGCACT CAAGTT-[TAMRA]). The probe was designed, using Primer Express software (Applied Biosystems), to lie across the splice junction of the unique *rrnO-lacZ* sequence, thus ensuring the assay was specific for vegetative cells of *B. subtilis* SC2362. The qRT-PCR assay was validated using the same RNA preparations as those used to validate the semiquantitative RT-PCR assay (i.e., those from control tissue spiked with known numbers of vegetative cells). Results were plotted to obtain a calibration curve for the qRT-PCR assay (Fig. 4). The efficiency of the qRT-PCR was low (55%) as an unfortunate consequence of having to specifically target the splice junction of the *rrnO-lacZ* sequence. However, considering that the qRT-PCR assay was used for absolute quantification of *B. subtilis* SC2362 vegetative cells (as opposed to relative quantification by comparison to an endogenous housekeeping gene) and that there was a good linear relationship between the $\log_{10}$(number of vegetative cells) and qRT-PCR signal strength ($R^2 = 0.9970$), the assay was fit for the purpose of this study. The absolute detection limit of the qRT-PCR assay was just 2 cells or 667 cells per gram of tissue. (Total RNA was extracted from sections of tissue weighing 30 mg and eluted in 50 μl. A 5-μl volume of eluate was then used as template in qRT-PCR.) Control reactions with no RNA template or RNA template prepared from vegetative cells of the parental *B. subtilis* strain PY79, spores of *B. subtilis* SC2362, or from unspiked (negative control) chick tissue did not yield any qRT-PCR signal, confirming that the assay was both robust and specific for the *rrnO-lacZ* mRNA of *B. subtilis* SC2362 vegetative cells.

Analysis of experimental samples from the 12- and 20-h time points by qRT-PCR enabled us to estimate the numbers of *B. subtilis* SC2362 vegetative cells present (Table 1). Furthermore, the enhanced sensitivity of the qRT-PCR assay enabled us to estimate the numbers of vegetative cells in samples which did not test positive by semiquantitative RT-PCR, most notably, samples of small intestine. Considering that spore doses were administered in sterile distilled water, which lacks any of the chemical or nutrient signals associated with spore germination (12, 23), this suggests that spore germination was induced either prior to or upon entry into the chick small intestine. Interestingly, the number of vegetative cells was found to surpass the number of spores between 12 and 20 h postdosing in all regions of the GI tract (Table 1). Based on the results of the present study, it is not possible to comment on whether this

TABLE 1. Numbers of *B. subtilis* SC2362 spores and vegetative cells recovered from GI tracts of chicks

Time postdosing (h)	Tissue	Chick	Spores/g of tissue[a]	Vegetative cells/g of tissue (qRT-PCR 40 − C_t value)[b]	Ratio of spores to vegetative cells
12	Small intestine	1	6.61×10^3	1.14×10^3 (1.39)*	5.80:1
		2	4.50×10^4	2.44×10^4 (8.38)*	1.84:1
		3	2.47×10^4	4.01×10^3 (4.26)*	6.16:1
	Ceca	1	2.47×10^7	1.64×10^6 (17.98)	15.06:1
		2	8.83×10^6	1.26×10^6 (17.38)	7.01:1
		3	1.03×10^7	6.82×10^4 (10.73)*	151.01:1
	Colon	1	1.18×10^6	5.04×10^5 (15.30)	2.34:1
		2	8.83×10^5	8.70×10^5 (16.54)	1.01:1
		3	2.08×10^5	2.40×10^4 (8.35)*	8.67:1
20	Small intestine	1	2.61×10^3	3.72×10^3 (4.09)*	1:1.43
		2	8.67×10^3	1.02×10^4 (6.40)*	1:1.18
		3	3.72×10^3	7.76×10^3 (5.77)*	1:2.09
	Ceca	1	1.77×10^6	4.55×10^7 (25.57)	1:25.71
		2	9.33×10^5	3.60×10^6 (19.78)	1:3.86
		3	1.40×10^6	2.31×10^6 (18.77)	1:1.65
	Colon	1	1.33×10^5	3.93×10^6 (19.98)	1:29.55
		2	1.63×10^5	1.58×10^6 (17.90)	1:9.69
		3	5.17×10^4	5.92×10^6 (20.92)	1:114.51

[a] Spore numbers at 12 and 20 h postdosing were determined by plating as described in the text.
[b] Vegetative cell numbers were determined by qRT-PCR. Experimental samples were analyzed in duplicate on two separate occasions. Vegetative cell numbers were extrapolated from mean (40 − C_t) values, using the equation which describes the qRT-PCR calibration curve (Fig. 4). * indicates samples which were beyond the detection limit of the semiquantitative RT-PCR assay.

was due to spore germination alone or whether cell replication within the host GI tract was also a contributing factor.

Retention of *B. subtilis* in the chick GI tract. Finally, estimates were made as to what percentage of the original spore dose (1×10^9) was retained in the GI tract of each chick at 12 and 20 h postdosing. To do this, the GI tissues of 12 1-day-old chicks were excised and weighed immediately postmortem. The mean weight of small intestines was 1.21 ± 0.12 g, that of ceca was 0.25 ± 0.10 g, and that of colons was 0.1 ± 0.01 g. Using these approximations in conjunction with plating (spores) and qRT-PCR (vegetative cells) data, estimates were made as to the numbers of spores and vegetative cells retained throughout the entire GI tract of each chick at 12 and 20 h postdosing. Between 0.13% and 1.23% of the original spore dose was estimated to have been retained in the GI tract of each chick (Table 2). This finding is comparable with that from work conducted in mice (6, 25) and supports the notion that *B. subtilis* is a transient member of the chick GI microflora, which does not colonize the GI tract. The remainder of the original spore dose may have been shed in feces or germinated in the GI environment and subsequently died due to unfavorable conditions.

TABLE 2. Retention of *B. subtilis* SC2362 in the GI tracts of chicks[a]

Time postdosing (h)	Chick	*B. subtilis* SC2362 spore or vegetative cell count (% of original spore dose)		
		Spores	Vegetative cells	Total
12	1	6.37×10^6 (0.64)	4.64×10^5 (0.05)	6.83×10^6 (0.68)
	2	2.36×10^6 (0.24)	4.34×10^5 (0.04)	2.79×10^6 (0.28)
	3	2.63×10^6 (0.26)	2.44×10^4 (0.002)	2.65×10^6 (0.27)
20	1	4.64×10^5 (0.05)	1.18×10^7 (1.18)	1.23×10^7 (1.23)
	2	2.61×10^5 (0.03)	1.07×10^6 (0.11)	2.33×10^6 (0.13)
	3	3.60×10^5 (0.04)	1.20×10^6 (0.12)	1.56×10^6 (0.16)

[a] Values presented are estimates based on approximated weights for the small intestine, cecum, and colon of a 1-day-old chick, as described in the text.

In conclusion, we have proven for the first time that spores of *B. subtilis* are able to germinate in the chicken GI tract. This principle finding agrees with studies conducted with a mouse model (6, 25). However, in those studies, vegetative cells were detected only in the small intestines of mice dosed with spores. In contrast, vegetative cells were detected throughout the GI tracts of chicks in the present study. This observation demonstrates that the findings of experiments conducted with a mammalian model may not translate directly to an avian host. Moreover, these findings may reflect genuine physiological and/or physicochemical differences between the GI tracts of chicks and mice. Notwithstanding these differences, it is noteworthy that chicks in the present study were 1 day old at the time of dosing, whereas mice used in the aforementioned studies were 6 weeks old at the time of dosing. Therefore, the state of GI microflora maturity and/or physiological status of the host may have played some part in the apparent disparity between chicks and mice. Nonetheless, the findings of the present study are considered highly relevant, as functional foods (e.g., probiotics) are typically administered to commercially farmed chickens from one day of age.

B. subtilis spore germination occurred rapidly in the GI tracts of chicks, with vegetative cells outnumbering spores 20 h after spore doses were administered. This may have been due to spore germination alone, although cell replication within the GI environment may also have been a contributing factor. Even though *B. subtilis* is classically considered to be an obligate aerobe, it may well survive and replicate within the anoxic GI environment, as it can use nitrate or nitrite (in place of oxygen) as a terminal electron acceptor during anaerobic respiration (20, 21).

The RT-PCR assays developed in the present study were

designed to identify *B. subtilis* SC2362 vegetative cells based on the presence of *rrnO-lacZ* mRNA. Considering that spore germination per se does not require de novo protein synthesis (19), the vegetative cells detected in this study must have either entered or completed the process of spore outgrowth, which is characterized by the onset of metabolic processes (23). This proves that although spore-based probiotics are metabolically dormant upon administration, they may germinate in the GI tract of chicks and function through mechanisms which require them to be metabolically active (e.g., secretion of antimicrobial compounds and/or competition for essential nutrients).

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REFERENCES

1. **Anadon, A., M. R. Martinez-Larranaga, and M. Aranzazu Martinez.** 2006. Probiotics for animal nutrition in the European Union. Regulation and safety assessment. Regul. Toxicol. Pharmacol. **45:**91–95.
2. **Balu, S., and P. Kaiser.** 2003. Avian interleukin-12beta (p40): cloning and characterization of the cDNA and gene. J. Interferon Cytokine Res. **23:**699–707.
3. **Bunyan, J., L. Jeffries, J. R. Sayers, A. L. Gulliver, and K. Coleman.** 1977. Antimicrobial substances and chick growth promotion: the growth promoting activities of antimicrobial substances, including fifty-two used either in therapy or as dietary feed additives. Br. Poult. Sci. **18:**283–294.
4. **Cartman, S. T., and R. M. La Ragione.** 2004. Spore probiotics as animal feed supplements, p. 155–161. *In* E. Ricca, A. O. Henriques, and S. M. Cutting (ed.), Bacterial spore formers: probiotics and emerging applications. Horizon Bioscience, Norfolk, United Kingdom.
5. **Cartman, S. T., R. M. La Ragione, and M. J. Woodward.** 2007. Bacterial spore formers as probiotics for poultry. Food Sci. Tech. Bull. **4:**21–30.
6. **Casula, G., and S. M. Cutting.** 2002. *Bacillus* probiotics: spore germination in the gastrointestinal tract. Appl. Environ. Microbiol. **68:**2344–2352.
7. **Coates, M. E., R. Fuller, G. F. Harrison, M. Lev, and S. F. Suffolk.** 1963. A comparison of the growth of chicks in the Gustafsson germ-free apparatus and in a conventional environment, with and without dietary supplements of penicillin. Br. J. Nutr. **17:**141–150.
8. **Duc le, H., H. A. Hong, T. M. Barbosa, A. O. Henriques, and S. M. Cutting.** 2004. Characterization of *Bacillus* probiotics available for human use. Appl. Environ. Microbiol. **70:**2161–2171.
9. **Duc le, H., H. A. Hong, N. Q. Uyen, and S. M. Cutting.** 2004. Intracellular fate and immunogenicity of *B. subtilis* spores. Vaccine **22:**1873–1885.
10. **FAO/WHO.** 2002. Guidelines for the evaluation of probiotics in food. Report of a joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) working group on drafting guidelines for the evaluation of probiotics in food. World Health Organization. http://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf.
11. **Forbes, M., and J. T. Park.** 1959. Growth of germ-free and conventional chicks: effect of diet, dietary penicillin and bacterial environment. J. Nutr. **67:**69–84.
12. **Foster, S. J., and K. Johnstone.** 1990. Pulling the trigger: the mechanism of bacterial spore germination. Mol. Microbiol. **4:**137–141.
13. **Hisanga, S.** 1980. Studies on the germination of genus *Bacillus* spores in rabbit and canine intestines. J. Nagoya City Med. Assoc. **30:**456–469.
14. **Hoa, N. T., L. Baccigalupi, A. Huxham, A. Smertenko, P. H. Van, S. Ammendola, E. Ricca, and A. S. Cutting.** 2000. Characterization of *Bacillus* species used for oral bacteriotherapy and bacterioprophylaxis of gastrointestinal disorders. Appl. Environ. Microbiol. **66:**5241–5247.
15. **Hoa, T. T., L. H. Duc, R. Isticato, L. Baccigalupi, E. Ricca, P. H. Van, and S. M. Cutting.** 2001. Fate and dissemination of *Bacillus subtilis* spores in a murine model. Appl. Environ. Microbiol. **67:**3819–3823.
16. **Hong, H. A., H. Duc le, and S. M. Cutting.** 2005. The use of bacterial spore formers as probiotics. FEMS Microbiol. Rev. **29:**813–835.
17. **Leser, T. D., A. Knarreborg, and J. Worm.** 2007. Germination and outgrowth of *Bacillus subtilis* and *Bacillus licheniformis* spores in the gastrointestinal tract of pigs. J. Appl. Microbiol. **104:**1025–1033.
18. **McCartney, E.** 2004. EU regulations on bacillary probiotics for animal feeds, p. 221–227. *In* E. Ricca, A. O. Henriques, and S. M. Cutting (ed.), Bacterial spore formers: probiotics and emerging applications. Horizon Bioscience, Norfolk, United Kingdom.
19. **Moir, A., B. M. Corfe, and J. Behravan.** 2002. Spore germination. Cell. Mol. Life Sci. **59:**403–409.
20. **Nakano, M. M., Y. P. Dailly, P. Zuber, and D. P. Clark.** 1997. Characterization of anaerobic fermentative growth of *Bacillus subtilis*: identification of fermentation end products and genes required for growth. J. Bacteriol. **179:**6749–6755.
21. **Nakano, M. M., and P. Zuber.** 1998. Anaerobic growth of a "strict aerobe" (*Bacillus subtilis*). Annu. Rev. Microbiol. **52:**165–190.
22. **Nicholson, W. L., and P. Setlow.** 1990. Sporulation, germination and outgrowth, p. 391–450. *In* S. M. Cutting and C. R. Harwood (ed.), Molecular biological methods for *Bacillus*. John Wiley & Sons Ltd., Chichester, United Kingdom.
23. **Setlow, P.** 2003. Spore germination. Curr. Opin. Microbiol. **6:**550–556.
24. **SSC for the European Commission, Health and Consumer Protection Directorate-General.** 1999. Opinion of the Scientific Steering Committee on antimicrobial resistance. http://ec.europa.eu/food/fs/sc/ssc/out50_en.pdf.
25. **Tam, N. K., N. Q. Uyen, H. A. Hong, H. Duc le, T. T. Hoa, C. R. Serra, A. O. Henriques, and S. M. Cutting.** 2006. The intestinal life cycle of *Bacillus subtilis* and close relatives. J. Bacteriol. **188:**2692–2700.
26. **Youngman, P., J. B. Perkins, and R. Losick.** 1984. Construction of a cloning site near one end of *Tn*917 into which foreign DNA may be inserted without affecting transposition in *Bacillus subtilis* or expression of the transposon-borne *erm* gene. Plasmid **12:**1–9.