

## REVIEW ARTICLE

**Do bacteria need to be regulated?**

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**Abstract**

Additives for use in animal nutrition are regulated under Regulation (EC) No. 1831/2003. The scope of this paper addresses the specific microbiological issues relevant to a microbial feed additive, containing a *Bacillus* spp. and uses as an example a product with the trade name, Calsporin®. *Bacillus subtilis* C-3102 is the active ingredient in Calsporin® and is added to animal feed to favourably affect animal production and performance (growth and feed efficiency), by modulating the gastrointestinal flora. It is not the purpose of this review to present the raw data for Calsporin® but rather to use Calsporin® as an example of the type of data required by the European regulatory authorities. At the time of preparation of this manuscript Calsporin® has yet to be reviewed by the authorities. The regulatory system under the auspices of the EFSA FEEDAP Panel is clearly attempting to move in line with development of scientific opinion and is to be applauded for such efforts. Bacteria do need to be regulated, and the regulations clearly provide adequate and appropriate protection to human health and to environmental considerations.

**Introduction**

Additives for use in animal nutrition are regulated under Regulation (EC) No. 1831/2003. The scope of Regulation (EC) No. 1831/2003 [Article 1(1)] 'is to establish a Community procedure for authorising the placing on the market and use of feed additives and to lay down rules for the supervision and labelling of feed additives and premixtures in order to provide the basis for the assurance of a high level of protection of human health, animal health and welfare, environment and users' and consumers' interests in relation to feed additives, whilst ensuring the effective functioning of the internal market'. Feed additives are defined under Regulation (EC) No. 1831/2003 [Article 2(2)] as 'substances, micro-organisms or preparations, other than feed material and premixtures, which are intentionally added to feed or water in order to perform, in particular, one or more of the functions mentioned in Article 5(3)'. Article 5(2) of this Regulation states that 'the feed additive shall not: (a) have an adverse effect on animal health, human health or the environment, (b) be presented in a manner which may mislead the user, and (c) harm the consumer by impairing the

distinctive features of animal products or mislead the consumer with regard to the distinctive features of animal products,' and that at Article 5(3) 'the feed additive shall: (a) favourably affect the characteristics of feed, (b) favourably affect the characteristics of animal products, (c) favourably affect the colour of ornamental fish and birds, (d) satisfy the nutritional needs of animals, (e) favourably affect the environmental consequences of animal production, (f) favourably affect animal production, performance or welfare, particularly by affecting the gastro-intestinal flora or digestibility of feeding stuffs, or (g) have a coccidiostatic or histomonostatic effect'.

The European Food Safety Authority (EFSA) provided a working guidance document for feed additives in September 2004 entitled, 'EFSA Administrative Guidance to Applicants on the Presentation of Applications for the Request of Authorisation of a Feed Additive'. The document was updated in July 2005 ([http://www.efsa.eu.int/science/feedap/authorisations/catindex\\_en.html](http://www.efsa.eu.int/science/feedap/authorisations/catindex_en.html)) and provides guidance on the presentation of applications for authorizing the placing and use of feed additives in the market, or for new use of a feed additive under community legislation [Regulation (EC) No. 1831/2003] to be

evaluated by the EFSA. This guidance was relevant for all applications submitted after 18 October 2004.

This paper addresses the specific microbiological issues relevant to a microbial feed additive, containing a *Bacillus* spp. and uses as an example a product with the trade name, Calsporin®. Calsporin® is added to animal feed to favourably affect animal production and performance (growth and feed efficiency), by modulating the gastrointestinal flora.

In addition to the overarching, 'EFSA Administrative Guidance to Applicants on the Presentation of Applications for the Request of Authorisation of a Feed Additive' there are a number of other relevant guidelines that sponsors need to be aware of. The Scientific Committee on Animal Nutrition (SCAN 2001) opinion is currently the recommended guideline for applications seeking authorization for micro-organisms as feed additives. This document shall hereafter simply be referred to as the SCAN Guideline. Additionally for products where the active component is a *Bacillus* spp., it is necessary to also consider the Opinion of the SCAN on the safety of use of *Bacillus* species in animal nutrition as expressed on 17 February 2000 (SCAN 2000).

A sponsor has to provide a full dossier for the product addressing the fundamental issues of quality, safety and efficacy. This paper only addresses the microbiology components with regard to safety and efficacy, although it is important to stress that for any microbiology product it is crucial that the sponsor demonstrates that the manufacturing process is sufficiently well controlled to ensure production of a uniform product that meets the agreed

specifications. The purpose of this paper is not to comment on the claims of Calsporin® but rather to review the regulatory requirements using Calsporin® as an example. In this context it is inappropriate to present the raw data described within this paper as it is the regulatory process that is being reviewed. As part of this process it is necessary to define a detailed product profile, it is this profile that describes the claims of the described product. Details of the Calsporin® product profile are shown in Table 1.

## Required data

### Nomenclature

The SCAN (2001) Guideline clearly states that the name and taxonomic status of the micro-organism must be provided in accordance with the latest published information in the 'International Codes of Nomenclature'. Microbiologists have recently been reminded of the importance of prokaryote nomenclature and the official indexing or registration system for the names of prokaryote taxa from subspecies up to, and including, class (Euzéby and Tindall 2004). The introduction of a variety of modern identification methods over the past 50 years now make it comparatively easy for scientists without training in systematics to approach the task of describing new taxa and as a result it becomes important to ensure that such descriptions are consistent with agreed taxonomic guidelines. Calsporin® is an interesting example because the taxonomy is not clear cut. The sponsor considers the product

**Table 1.** Details of the Calsporin® product profile

Proprietary name	Calsporin®
Type of additive	Calsporin® is a zootechnical feed additive, classified as a gut flora stabilizer (micro-organism)
Qualitative and quantitative composition	The Calsporin® product is a preparation of viable spores of <i>Bacillus subtilis</i> C-3102, at a concentration of $1 \times 10^{10}$ CFU g <sup>-1</sup> product, in a calcium carbonate carrier
Physical form of the product	The product is presented as a dry powder
Conditions of use	Calsporin® is added to feed as a feed additive at an inclusion rate of $1 \times 10^9$ CFU kg <sup>-1</sup> of complete feed
Target animal species	Chickens for fattening (broilers) until slaughter
Indications for use	As a zootechnical feed additive to improve performance (growth and feed efficiency)
Method of administration	In feed at a recommended dose of 100 ppm (100 g product per metric tonne of feed)
Mode of action	Calsporin® is a feed additive based on viable spores of <i>Bacillus subtilis</i> C-3102. It is fully accepted that the gastrointestinal tract is a complex ecosystem and as such the mode of action of microbial additives remains to be fully elucidated. There are multiple ways in which additives based on live micro-organisms (probiotics) may act. Published evidence suggests that Calsporin® reduces harmful, disease-causing bacteria in the gastrointestinal tract and zoonotic organisms of public health concern, and may increase beneficial lactobacilli (Maruta <i>et al.</i> 1996; Fritts <i>et al.</i> 2000). In simple terms the harmful bacteria are considered to cause damage to the gut wall, making it less able to absorb nutrients. It is likely that, in common with other bacillary probiotics, administered viable spores of <i>Bacillus subtilis</i> C-3102 help to maintain a beneficial microbial population balance within the gastrointestinal tract of the host animal, thus supporting efficient digestion, and so improving growth and feed efficiency

strain to be *Bacillus subtilis* with the strain suffix C-3102. *Bacillus subtilis* is clearly a valid bacterial name and as such it is important to examine the evidence that the product strain is indeed *B. subtilis*.

As a starting point it is relevant to consider the opinion of SCAN (2000) with respect to taxonomy of *Bacillus* spp. as expressed in their report of 17 February 2000. They made the point that bacteria that differentiate into endospores under aerobic conditions have traditionally been placed in the genus *Bacillus* and that over the past three decades this genus has expanded to accommodate more than 100 species. Analysis of 16S ribosomal RNA sequences from numerous *Bacillus* species has indicated that the genus *Bacillus* should be divided into at least five genera or rRNA groups (Ash *et al.* 1991). Since the work of Ash *et al.* (1991) and the subsequent isolation of many new species, SCAN (2000) reported that this number of 'genera' had increased to about 16. Within this framework, *B. subtilis*, the type species, is accommodated in rRNA group 1 or *Bacillus sensu stricto*.

Again quoting from the SCAN (2000) report, the *B. subtilis* group traditionally comprises four species: *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus pumilus* and *B. subtilis*. Recent ecological studies, however, have identified some very close relatives of *B. subtilis*: *Bacillus atrophaeus*, *Bacillus mojavenensis* and *Bacillus vallismortis* and have subdivided *B. subtilis* into subsp. *subtilis* and subsp. *spizizenii*. These taxa all conform to the DNA hybridization guidelines for bacterial species in that delineation of a bacterial species require strains within a species to share more than 70% chromosomal DNA hybridization and between species <70% hybridization. The 16S rRNA gene sequences differ between representative species of the *B. subtilis* group, but such data are not available for the recently described 'ecological' group. Species of the traditional group can be distinguished phenotypically, but *B. mojavenensis*, *B. subtilis* and *B. vallismortis* are indistinguishable and can only be identified by molecular means while *B. atrophaeus* is distinguished from *B. subtilis* only by pigmentation on media containing an organic nitrogen source (Nakamura 1989). The acceptance by the SCAN (2000) report of the inability to distinguish the members of the ecological group is a point to which we shall return. The evidence that the Calsporin® strain is indeed a strain of *B. subtilis* was supported by two independent reports, a critical evaluation of recent literature publications relating to *Bacillus* spp. taxonomy and additional supporting data regarding amylase production by the type Calsporin® strain.

The Biolog MicroStation™ system, designed for rapid identification of aerobic bacteria, anaerobic bacteria and yeasts provides a biochemically based identification by virtue of utilizing 95 proprietary carbon source utilization

tests in a microtitre plate format. The resulting characteristic pattern of reactions is compared with a database for species identification. Positive identification is achieved when the similarity index describing the relationship between test strain and database profile exceeded pre-set values within the system software. Based on the difference in similarity index between the stated identity and the next most likely identity, the MicroStation™ software was able to provide an overall probability (%) that the stated identity is correct. Biochemical identification of the test strain C-3102 using the Biolog MicroStation™ system confirmed the test strain to be a member of the *B. subtilis* taxon. Examination of the raw data showed that the test strain was identified as *B. subtilis* with 99% probability and a similarity index of 0.522. The second most likely identification was *B. amyloliquefaciens*, this had a 1% probability and a similarity index of 0.002.

16S rDNA gene sequence analysis of the test strain was also available for review. Approximately 95% of the 16S rRNA gene sequence was determined by direct sequencing of PCR-amplified 16S rDNA. Genomic DNA extraction, PCR-mediated amplification of the 16S rDNA and purification of the PCR product was carried out and purified PCR products sequenced using the CEQTMDCS-Quick Start Kit (Beckmann Coulter, Fullerton, CA, USA). Sequence reactions were electrophoresed using the CEQTM8000 Genetic Analysis System (Beckmann Coulter) and resulting sequence data was compared with representative 16S rRNA gene sequences of organisms belonging to the *Firmicutes*. The complete 16S rRNA gene sequence of the test strain showed highest similarity of 99.7% to *B. subtilis* ssp. *subtilis* and *B. amyloliquefaciens*. It was concluded that on the basis of this analysis the test strain might represent a strain of one of these species although it was pointed out that phylogenetic analysis revealed that it grouped with *B. amyloliquefaciens*. RiboPrinter analysis of strain C-3102 using the restriction enzyme *EcoRI* was carried out and strain C-3102 identified by the DuPont Identification Library as *B. subtilis*. The C-3102 strain was also compared with the RiboPrint patterns of other *Bacillus* strains lodged in the DSMZ German Collection of Microorganisms and Cell Cultures and it was shown that the highest similarity (0.94) was with a strain of *B. amyloliquefaciens* DSM 1062. The pattern of strain C-3102 showed a higher similarity to the type strain *B. amyloliquefaciens* DSM 7T (0.59) than to the type strain *B. subtilis* DSM 10T (0.26). On this basis it was concluded that strain C-3102 should be considered as a strain of the species *B. amyloliquefaciens*.

The biochemical and molecular identifications are consistent, yet there is clearly an issue as to whether strain C-3102 was correctly identified as *B. subtilis* or *B. amyloliquefaciens*. A recent publication from Reva *et al.* (2004) becomes important in helping to clarify the issues. They

made the important point that recent taxonomic studies have shown *B. subtilis* to be a heterogeneous group and should be considered as a complex of closely related species, as discussed previously. They also make the very pertinent point that *B. subtilis* and *B. amyloliquefaciens* are phenotypically similar species and can easily be confused. It is therefore not surprising that most large-scale studies of the endophytic microflora refer to isolates as *B. subtilis* without deeper analysis of their taxonomy. Reva *et al.* (2004) comment that definitive identification is important because biocontrol and plant promotion activities including antibacterial and antifungal polypeptide production, and synthesis of extracellular phytase and chitinase, are strain specific and may be associated with certain species and subspecies of *B. subtilis sensu lato*. This is an important point in relation to the data concerning production of amylase by strain C-3102 and in attempting to understand the RiboTyping data. It is inappropriate in this paper to detail all the issues pertaining to the challenges in *Bacillus* taxonomy and as such I will simply use one example and relate this to the RiboTyping study. Reva *et al.* (2004) point out that the well-characterized  $\alpha$ -amylase genes of *B. subtilis* and *B. amyloliquefaciens* have been used for distinguishing strains of these closely related species and so they amplified this gene using two pairs of standard primers ABa1/ABa2 and ABs1/ABs2 specific for the  $\alpha$ -amylase genes of *B. amyloliquefaciens* and *B. subtilis* respectively. It is relevant to note that primers ABa1/ABa2 produced a product of the correct size with DNA from *B. amyloliquefaciens* strain DSM 7<sup>T</sup> but failed with all the plant-associated strains of *B. amyloliquefaciens*. The reason for highlighting *B. amyloliquefaciens* strain DSM 7<sup>T</sup> is that this was one of the strains cited in the RiboPrinting study and which had a close similarity to the test strain C-3102, yet in the studies of Reva *et al.* (2004). *Bacillus amyloliquefaciens* strain DSM 7<sup>T</sup> was clearly different to all the other tested plant-associated strains of *B. amyloliquefaciens*. There may thus be some question as to whether *B. amyloliquefaciens* strain DSM 7<sup>T</sup> is indeed appropriately identified. What is beyond refute is that strain C-3102 is either *B. amyloliquefaciens* or *B. subtilis*. The most compelling evidence that strain C-3102 is distinct from *B. amyloliquefaciens* and can thus be considered to be truly *B. subtilis* was found in a study that compared amylase production in isolates of *B. amyloliquefaciens* (10 strains), *B. subtilis* (six strains) and three incompletely or unidentified strains, including strain C-3102. The *B. subtilis*-type amylase gene is highly preserved within the species of *B. subtilis*, but is less common in other species. PCR analysis was carried out using specific primers for detection of *B. amyloliquefaciens*-type and *B. subtilis*-type amylase genes, respectively. The amplified product was confirmed by electrophoresis and sequence analysis

performed on unexpected amplified products for further confirmation. A genetic probe was constructed from the *B. amyloliquefaciens*-type amylase gene, obtained by PCR amplification of *B. amyloliquefaciens* strain CP799. Southern hybridization was performed on the bacterial genome, which was digested by restriction enzymes. The *B. amyloliquefaciens*-type amylase-specific band was detected in all strains of *B. amyloliquefaciens*, but not in other strains. The *B. subtilis*-type amylase-specific band was detected in all strains of *B. subtilis*, *B. amyloliquefaciens* strain CP815 and in *B. subtilis* strain C-3102, but not in other strains. It was thus clearly shown that *B. subtilis* strain C-3102 has a *B. subtilis*-type amylase gene that was distinct from the *B. amyloliquefaciens*-type amylase gene. As all 10 strains of *B. amyloliquefaciens* used in the study were shown to have the *B. amyloliquefaciens*-type amylase gene, it was concluded that *B. subtilis* strain C-3102 has a closer relationship to *B. subtilis* than to *B. amyloliquefaciens*.

### Biological origin

Sponsors are required to state the origin of the test strain. *Bacillus subtilis* C-3102 was derived from the soil in Japan. Additionally the SCAN Guideline requires the test strain to be deposited in two internationally recognized culture collections. Deposition certificates need to be included in the dossier.

Studies then need to be reported that describe the relevant morphological, physiological, and molecular characteristics necessary for identification of the strain and which confirm its genetic stability. It is necessary to provide extensive data showing the growth and survival characteristics of the test organism under different conditions of pH, temperature and oxygen. In the Calsporin® example the sponsor additionally provided data relating to colonial morphology under different atmospheric conditions. *Bacillus subtilis* C-3102 was shown to be able to survive from 4 to 60°C, but not at 80°C, and to grow at a range of temperatures from 20 to 50°C. This is an interesting point and one that requires further comment. Whilst it is indeed correct that *B. subtilis* C-3102 failed to survive at 80°C it is important to make the point that this study was carried out in a broth medium as would be appropriate for such a study. Additional studies relating to the manufacturing process show that *B. subtilis* C-3102 will survive temperatures of 90°C within the animal feed pelleting process. This demonstrates an important point with regard to study design and is fundamental to the issues faced by applied microbiologists. It is crucial that the correct questions are posed when designing study protocols. The question here is not simply what is the temperature tolerance of *B. subtilis* C-3102 but rather what is the temperature tolerance under defined conditions. The conditions need to be defined

relative to the appropriate process, in this case it is the pelleting of animal feed into which the described probiotic is to be incorporated. With respect to environmental pH, survival of *B. subtilis* C-3102 was detected from pH 3.0 to 11.0 and growth from pH 5.0 to 9.0. It was shown that oxygen was required for optimal growth of *B. subtilis* C-3102 and that growth yield was lower in an anaerobic atmosphere. In comparison with the test organism grown aerobically, anaerobic colonies were smaller, yet microaerophilic colonies were larger. Spread, overlaid and pour plating methods of *B. subtilis* C-3102 resulted in similar growth efficiency, with similar viable counts. *Bacillus subtilis* C-3102 when grown aerobically in pure culture on nonselective media at 37°C on spread plates, forms greyish white, conical colonies (characteristically described as 'mountain shaped') with granular, dry surfaces, containing a sticky, translucent substance inside. No major differences were observed in colonial morphology between spread and overlaid culture methods, whereas typical variations were observed by the pour plate method, in that submerged colonies lost the characteristic mountain shape and become smoother in borders and surfaces, also losing their surface granularity.

Phenotypic and genetic stability of the test organism and any demonstration of plasmid carriage must be described. Phenotypic stability for Calsporin® was studied by determining colony morphology, cell characteristics, motility and biochemical parameters. Genetic stability was studied by determining the pulsed-field gel electrophoresis profile and the frequency of spontaneous mutation in the presence of antibiotics, rifampicin and streptomycin. These were selected as genes resistant to these two antibiotics are commonly used as indicators of spontaneous mutation (Miller 1992). The results obtained for *B. subtilis* C-3102 clearly showed stability of *B. subtilis* C-3102 under the stress conditions studied and across time. To screen for presence of plasmids, DNA was extracted from overnight cultures of *B. subtilis* C-3102 using two different methods and analysed by agarose gel electrophoresis. The results clearly indicated that *B. subtilis* C-3102 did not harbour plasmids. The results were consistent irrespective of the method of DNA extraction used in the study.

Sponsors need to make a clear statement that the test organism has no genetic modification as defined by Article 2 of Council Directive 2001/18/EC6. If indeed the organism were genetically modified then the sponsor would need to meet all the requirements demanded by the respective regulation.

### Toxins and virulence factors

The SCAN (2000) Guideline is very clear that strains of micro-organism belonging to the *Bacillus* genus and thus

known to be capable of producing toxins should be subject to appropriate tests to demonstrate at a molecular and cellular level the absence of any cause for concern. Within the guideline it makes the important point that the incidence of food poisoning involving strains of the *B. cereus* taxonomic group is common and that cases where the causative organism is a strain of *Bacillus* other than a member of the *B. cereus* group are rare. Following this logic the guideline draws a number of conclusions:

- i Probably all members of the *B. cereus* taxonomic group are toxin producers, although large differences occur in the nature and amount of toxins produced. It considers that all strains of this group probably pose a hazard in the human food chain.
- ii There is no taxonomic difference between *B. cereus* and *B. thuringiensis* other than the presence of plasmids encoding delta-endotoxins in the latter. From the viewpoint of safety assessment they should be considered to belong to the same species.
- iii Strains of the *B. cereus* group associated with outbreaks of food poisoning generally produce higher concentrations of toxins in laboratory cultures than those isolated from other sources.
- iv Strains from six species, three from the *B. subtilis* group (*B. subtilis*, *B. licheniformis* and *B. pumilus*) and three from other groups (*Bacillus alvei*, *Bacillus circulans* and *Bacillus sphaericus*) also have been implicated in food poisoning. Although little is known about their virulence factors, both emetic and enterotoxin(s) must be involved. Within the *B. subtilis* group there are strains that pose a similar hazard to those in the *B. cereus* group. However, a majority of strains evidently lack the gene(s) encoding toxin(s) or if present, a capacity for toxin production or fail to produce detectable levels of toxin under the conditions employed, and these could be considered safe for use.
- v At this time insufficient is known about the genetic basis for toxin production in *Bacillus* species to rely wholly on PCR-based evidence of the absence of a toxigenic potential. Identification of hazard will continue to depend on the detection of the toxin(s) and/or their effects on biological systems.

The SCAN Guideline thus went on to make the following recommendations:

- i It is evident that a substantial majority, if not all, strains of *Bacillus cereus* and closely related species produce toxins that may be damaging to human health.
  - a. SCAN recommends that, for all future applications involving the addition of living organisms to animal feeds, the use of strains from the *B. cereus* taxonomic group be strongly discouraged.
  - b. For additives based on strains belonging to the *B. cereus* taxonomic group with an existing history of use,

or for new products where benefits of use can be shown to substantially outweigh any risks, the best available methods should be used to demonstrate the absence of toxin production. A scheme for testing for the presence of toxins in strains of bacilli is proposed in the Annex to the Guideline.

c. As knowledge of toxin production amongst strains of *Bacillus* is incomplete, it is further recommended that any authorization for use granted should be subject to periodic review and that, in the light of any new and relevant information, additional testing should be required.

ii SCAN recognized that the risks posed by strains of *Bacillus* other than those from the *B. cereus* taxonomic group are less severe and that, with the exception of strains from the *B. subtilis* taxonomic group, detection of toxin production is the exception. Accordingly, provided toxin production cannot be detected, products based on species of *Bacillus*, other than those from the *B. cereus* taxonomic group, should be accepted. SCAN recommends the use of the scheme for testing proposed in the Annex to the Guideline.

iii For enzyme production or for other products of *Bacillus* species which do not constitute the whole organism, the producing strain should ideally be shown not to produce toxins under production conditions. However, where low levels of toxins are produced, detectable only after concentration of the culture medium, SCAN recognizes it may be possible to introduce adequate monitoring to ensure that the product itself is free from toxins or live organisms.

In the Guideline SCAN draws to the Commission's attention the widespread use of certain species of *Bacillus* as plant biopesticides. Several *Bacillus* species are registered as pesticides (see <http://www.epa.gov/pesticides/biopesticides/>) elsewhere in the world, but in Europe only strains of *B. thuringiensis* are presently used for this purpose. *Bacillus thuringiensis* is essentially indistinguishable from *B. cereus* and virtually all strains tested produced enterotoxins. If the Commission adopts SCAN's recommendations for the more stringent testing of *Bacillus* strains used as animal feed additives because of the risk associated with the indirect contamination of animal products, then authorization for the direct application of living strains of *B. thuringiensis* to human food crops would seem to require at least the same degree of safety assurance.

Following the SCAN recommendations the *B. subtilis* C-3102 strain was shown not to be cytotoxin positive, neither did it produce any toxins that cross-reacted with antibodies against NheA or Hb1C. The genes for *hbl* and *nhe* and *cytK* were not detected by PCR and the sperm motility test was negative. It was concluded that no

known toxic properties could be detected from the *B. subtilis* C-3102 strain.

It is interesting to note that the Hep2 test is within the SCAN-recommended scheme for testing *Bacillus* strains although if sperm motility test data are available then emetic toxin detection will be covered. The sperm assay is now better understood and documented relative to when the SCAN opinion was written. It is pertinent to note that since the publication of the SCAN (2000) Opinion, *Bacillus*-containing microbial additives have been approved in Europe for which sperm motility rather than Hep2 data have been provided within the dossier. This is a clear example where the regulatory authorities are prepared to acknowledge that our understanding of the science moves faster than the regulatory framework which by essence will inevitably always be responding to such changes.

### Antibiotic production and antibiotic resistance

#### *Antibiotic production*

The SCAN Guideline clearly states that micro-organisms intended as active agents should not be capable of producing antimicrobial substances relevant to the use of antibiotics in humans or animals. This can be a somewhat contentious issue, as unlike some of the other parameters, there is no clear guidance as to an appropriate testing strategy.

In the absence of direct guidance some sponsors have based assays on the FAO (1999) protocol using a range of tester strains including *B. cereus*, *Staphylococcus aureus*, *Serratia marcescens*, *Escherichia coli*, *B. circulans* and *Streptococcus pyogenes*. The assay is purely qualitative and results considered positive if a clear zone was observed around the test aliquot. The difficulties in interpreting these data are that according to the original protocol on which the study was based (FAO 1999) the result should only be considered positive when a minimum of three organisms are inhibited. A clear inhibition zone, less than the 16 mm diameter defined in the FAO protocol is indicative of a positive test. On this basis *B. subtilis* C-3102 can be considered as negative with respect to antibiotic production.

#### *Antibiotic resistance*

Strains of bacteria intended for use as an additive should not contribute to the reservoir of antibiotic resistance genes already present in the gut flora of animals and the environment and thus it is necessary to screen the test organism for resistance against representatives of the antibiotic families in use in human and veterinary medicine. This is an area in which the guideline has recently been revised (EFSA 2005).

The issues are summarized in the statement, 'The emergence and the spread of resistance to antibiotics in bacteria poses a threat to public and animal health and presents a major financial and societal cost'. In an effort to slow the development of resistance various actions have been taken at Community level, including, from the beginning of January 2006, the removal of all antibiotics used for growth promotion purposes from animal feed. With this objective, the SCAN adopted an opinion in July 2001 defining the criteria used to assess the presence or absence of resistance determinants to antibiotics in microbial feed additives.

This opinion has been revised (EFSA 2005) taking into consideration the data published after the adoption of this opinion to define appropriate breakpoint values as indicative of the need for a more extensive assessment of the basis for the resistance and to consider whether the distinction between 'intrinsic' and 'acquired' resistance used as indicative of the probability of transfer of resistance is still valid for the safety assessment of microbial feed additives.

Resistance to a given antibiotic can be inherent to a bacterial species or genus (intrinsic or natural resistance), while acquired resistance can be due either to added genes (genes acquired by the bacteria via gain of exogenous DNA) or to the mutation of indigenous genes. Chromosomal mutations can result in an increased resistance to antibiotics mainly by modifying the antibiotic-binding site, or by altering the expression of either intrinsic resistance determinants or the target molecule itself, which may overcome total inhibition by the drug.

The transfer of resistance to human or animal pathogenic bacteria which could result from the use of microbial products is related to the genetic basis of resistance. Although it is reasonable to assume that gene transfer from viable micro-organisms will occur to other micro-organisms in open environments such as the gastrointestinal tract, intrinsic resistance and genomic mutations are presumed to present a minimal potential (in practice no potential) for spread, whereas acquired resistance mediated by added genes, are considered as having a high potential for spread. Criteria for identifying bacterial strains with acquired resistance to antibiotics have been defined. For the purpose of distinguishing strains harbouring acquired antibiotic resistances from susceptible strains, the FEEDAP Panel defines microbiological breakpoints.

Microbiological breakpoints are set by studying the distribution of minimal inhibitory concentration (MICs) of the 13 considered antibiotics in bacterial populations belonging to a single taxonomical unit (species or genus). The part of the population that clearly deviates from the normal susceptible populations is categorized as resistant.

The data used for the definition of microbiological breakpoints were derived from the body of research published and from national and European monitoring programmes.

The detection of the MIC above the breakpoint levels as identified by the Panel, for one or more antibiotics requires further investigations to make the distinction between acquired and intrinsic resistance. Where all, or virtually all, strains within a given taxonomic group show phenotypic resistance to an antibiotic, that resistance can be considered intrinsic to the taxonomic group. Resistant strains belonging to a taxonomic group usually susceptible to an antibiotic are considered to harbour acquired antimicrobial resistance.

The FEEDAP Panel considers that strains of bacteria carrying an acquired resistance to antibiotics used in human and veterinary medicine should not be used in microbial feed additives, unless it can be proved that the genetic basis of the resistance is due to chromosomal mutation. It is thus relevant to include the breakpoints (Table 2) that will be used to determine the significance of the resistance profile of the test strain.

From the assessment of the current scientific data, it has been concluded that:

- i Where all, or virtually all, the strains within a given species (other taxonomic units) show resistance to an antibiotic, that resistance can be considered intrinsic to the taxonomic group. Provided that the gene (or genes)-conferring resistance is (are) not associated with mobile genetic elements, the risk of transfer to other organisms can be considered for practical purposes to be zero.
- ii Where resistance has been acquired by a strain belonging to a taxonomic group usually susceptible to an antibiotic, then the degree of risk of transfer generally is considered to be substantially greater than that associated with intrinsic resistance, unless it can be shown that the genetic basis of the resistance is due to chromosomal mutation.
- iii It is considered that strains of bacteria carrying an acquired resistance to antibiotics used in human and veterinary medicine should not be used in microbial feed additives.

On the basis of the generated data no antibiotic resistance was identified in the Calsporin® test strain and as a consequence no further work regarding antibiotic resistance was required by the SCAN guideline.

#### **Effects on the microflora of the digestive tract**

The guideline advises that in most cases, it is sufficient to limit such studies to the enumeration of microbial groups that can be routinely cultivated from the faecal flora and have safety implications, thus including data with respect

**Table 2** Microbiological breakpoints categorizing bacteria as resistant ( $\text{mg l}^{-1}$ ). Strains with MICs higher than the breakpoints below are considered as resistant (EFSA 2005)

	<i>Lactobacillus</i> obligate homofermentative	<i>Lactobacillus</i> heterofermentative*	<i>Lactobacillus plantarum</i>	<i>Enterococcus</i>	<i>Pediococcus</i>	<i>Leuconostoc</i>	<i>Lactococcus lactis</i>	<i>Streptococcus thermophilus</i>	<i>Bacillus</i> spp.	Other Gram+
Ampicillin	4	4	4	8	4	4	4	4	n.r.	2
Vancomycin	4	n.r.	n.r.	8	n.r.	n.r.	4	4	4	4
Gentamicin†	8	8	64	512	4	4	8	8	4	4
Kanamycin†	16	16	64	1024	4	8	8	8	8	8
Streptomycin†	16	16	64	1024	4	8	16	16	8	8
Neomycin†	16	16	32	1024	8	8	8	8	8	8
Erythromycin	4	4	4	4	4	4	4	4	4	4
Clindamycin	4	4	4	4	4	4	4	4	4	4
Quinupristin + dalbopristin	4	4	4	4	4	4	4	4	4	4
Tetracycline	8	8	32	16	4	4	4	4	8	4
Chloramphenicol	4	4	8	8	4	4	8	8	8	4
Trimethoprim†	8	8	8	8	8	8	n.r.	n.r.	8	8
Linezolid	4	4	4	4	4	4	4	4	4	4

n.r., not required.

\*Including *L. salivarius*.

†Possible interference of the growth medium.

to opportunistic pathogens including coliforms, enterococci and clostridia. The provided data for Calsporin® showed that administration of *B. subtilis* C-3102 does not have any adverse effect on the normal flora of poultry, as defined within the context of the guideline. This opinion is supported by published data (Maruta *et al.* 1996; Fritts *et al.* 2000), both studies indicating that Calsporin® is helpful in suppressing enteropathogens in broilers under challenge or field conditions.

The guideline also requires the sponsor to provide data regarding intestinal survival and rate of disappearance from faeces after withdrawal of the particular strain used in the product. As there was no claim for pathogen control for Calsporin® there was no need for data on the excretion of human pathogens. The determination of the disappearance of the test organism from the faeces is not a straightforward study as it is crucial to separate birds according to treatment phase. In the case of Calsporin® day-old birds were treated and on day 20, 96 birds from the Calsporin® treatment were relocated from the efficacy facility to a separate facility and received control diets until day 38. The experimental design was appropriately robust as the only *B. subtilis* C-3102 to be brought into the facility were those organisms resident in the gastrointestinal tract of the treated birds. Faecal samples were taken from the birds in both groups on days 20, 23, 27 and 30 and screened for *B. subtilis* C-3102. The data clearly showed that at the point of withdrawal (day 20) of Calsporin® from the diets, the faeces of the birds receiving the Calsporin® diet had a significantly higher *B. subtilis* count than the control group, approx.  $2 \log_{10} \text{g}^{-1}$  count difference. Three days after withdrawal of the treatment the numbers of *B. subtilis* in the positive treatment group were equivalent to those of the control group and remained so for the remainder of the study. The data clearly demonstrate that numbers of *B. subtilis* can only be maintained at elevated levels within the gastrointestinal tract of poultry by continued administration of *B. subtilis* C-3102. These data thus address both questions relating to intestinal survival and rate of disappearance from faeces. *Bacillus subtilis* C-3102 clearly does not colonize the broiler intestinal tract permanently and is only maintained in the gut by continuous intake, disappearing from the gastrointestinal tract 3 days after withdrawal from feed.

### Other issues

The only remaining issues that require microbiological review are to ensure that there are no potential incompatibilities with other feed ingredients, other approved additives or with medicinal products added to medicate animal feeds. In this case performance as measured by

growth, feed intake and feed efficiency will be tested against standard diet formulations. Within such studies it is necessary to ensure that the numbers of the test organism found in faeces of the birds fed diets supplemented with the test product on trial demonstrated normal establishment in the gut of the target animals. In the case of Calsporin® the counts of *B. subtilis* C-3102 in the feeds were as expected. In all the reviewed Calsporin® studies it was very clear that administration of Calsporin® leads to an increase in *B. subtilis* counts and that withdrawal of the product results in a concomitant decrease in *B. subtilis* count to that of the negative control group of animals.

## Conclusion

Bacteria are regulated and the regulations clearly provide adequate and appropriate protection to human health and to environmental considerations. This paper has reviewed the data for *B. subtilis* C-3102, the active ingredient in Calsporin®, at the time of preparation of this manuscript Calsporin® has yet to be reviewed by the authorities. The regulatory system under the auspices of the EFSA FEEDAP Panel is clearly attempting to move in line with development of scientific opinion and is to be applauded for such efforts. Bacteria do need to be regulated; the public can have confidence that they are being effectively regulated.

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