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# **Final Screening Assessment for *Bacillus circulans* strain ATCC 9500**

**Environment and Climate Change Canada**  
**Health Canada**

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**Canada** 

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## Synopsis

Pursuant to paragraph 74(b) of the Canadian Environmental Protection Act, 1999 (CEPA), the Minister of the Environment and the Minister of Health have conducted a screening assessment on *Bacillus circulans* strain ATCC 9500.

*B. circulans* strain ATCC 9500 is a bacterium that has characteristics in common with other strains of the species *B. circulans*. *B. circulans* is an endospore-forming bacterium that is present in many environments. It has been isolated from soils and marine water, and is found in association with plants and animals. *B. circulans* has properties that make it of potential use in aquaculture, bioremediation, biodegradation, water and wastewater treatment, drain cleaning and degreasing and enzyme production.

There has been no adverse effect attributed to *B. circulans* strain ATCC 9500 in the environment. However, in the context of experimental investigations into their biocontrol potential, some strains of *B. circulans* have shown pathogenic potential towards some insects and nematodes when directly inoculated with high concentrations. Nonetheless, *B. circulans* species is not considered a plant or animal pathogen, and in spite of its widespread distribution in the environment, there is no evidence that *B. circulans* has adversely affected terrestrial invertebrates at the population level.

There have been no human infections attributed to *B. circulans* strain ATCC 9500, and as a species, *B. circulans* is not known as a human pathogen. Despite its ubiquity, there has been few case reports of human infection with *B. circulans*, and these occurred mostly in individuals with pre-existing health conditions. *B. circulans* strain ATCC 9500 is sensitive to different classes of antibiotics including aminoglycosides, glycopeptides, second-generation fluoroquinolones and third-generation cephalosporins which may be used in the unlikely event of infection with this organism.

This assessment considers the aforementioned characteristics of *B. circulans* strain ATCC 9500 with respect to environmental and human health effects associated with consumer and commercial product use and in industrial processes subject to CEPA, including releases to the environment through waste streams and incidental human exposure through environmental media. To update information about current uses, the Government launched a mandatory information-gathering survey under section 71 of CEPA, as published in the Canada Gazette, Part I, on October 3, 2009 (section 71 notice). Information submitted in response to the section 71 notice indicates that *B. circulans* strain ATCC 9500 of this microorganism is used in consumer and commercial products for biodegradation, drain cleaning and degreasing, septic tank maintenance, as well as waste and wastewater treatment.

Based on the information available, it is concluded that *B. circulans* strain ATCC 9500 does not meet the criteria under paragraph 64(a) or (b) of CEPA as it is not entering the environment in a quantity or concentration or under conditions that have or may have an immediate or long-term harmful effect on the environment or its biological diversity or that constitute or may constitute a danger to the environment on which life depends. It is

also concluded that *B. circulans* strain ATCC 9500 does not meet the criteria under paragraph 64(c) of CEPA as it is not entering the environment in a quantity or concentration or under conditions that constitute or may constitute a danger in Canada to human life or health.

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## Introduction

Pursuant to paragraph 74(b) of the Canadian Environmental Protection Act, 1999 (CEPA), the Minister of the Environment and the Minister of Health are required to conduct screening assessments of living organisms added to the Domestic Substances List (DSL) by virtue of section 105 of the Act to determine whether they present or may present a risk to the environment or human health (according to criteria set out in section 64 of CEPA)<sup>1</sup>. *B. circulans* strain ATCC 9500 was added to the DSL under subsection 25(1) of CEPA 1988 and the DSL under subsection 105(1) of CEPA because it was manufactured in or imported into Canada between January 1, 1984 and December 31, 1986.

This screening assessment considers hazard information obtained from the public domain and from unpublished research data generated by Health Canada<sup>2</sup> and Environment and Climate Change Canada<sup>3</sup> research scientists, as well as comments from scientific peer reviewers. Exposure information was obtained from the public domain and from a mandatory CEPA section 71 notice published in the Canada Gazette, Part I, on October 3, 2009. Further details on the risk assessment methodology used are available in the Risk Assessment Framework document “Framework on the Science-Based Risk Assessment of Micro-organisms under the Canadian Environmental Protection Act, 1999” (Environment Canada and Health Canada 2011).

In this report, data that are specific to DSL-listed strain *B. circulans* strain ATCC 9500 are identified as such. Where strain-specific data were not available, surrogate information from literature searches was used. When applicable, literature searches conducted on the organism included its synonyms, and common and superseded names. Surrogate organisms are identified in each case to the taxonomic level provided by the source. Literature searches were conducted using scientific literature databases (SCOPUS, CAB Abstracts, Google Scholar and NCBI PubMed), web searches, and key search terms for the identification of human health and environmental hazards. Information identified up to October 2014 was considered for inclusion in this screening assessment report.

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<sup>1</sup> A determination of whether one or more criteria of section 64 of CEPA are met is based on an assessment of potential risks to the environment and/or to human health associated with exposure in the general environment. For humans, this includes, but is not limited to, exposure from air, water and the use of products containing the substances. A conclusion under CEPA may not be relevant to, nor does it preclude, an assessment against the criteria specified in the *Hazardous Products Regulations*, which is part of the regulatory framework for the Workplace Hazardous Materials Information System, 2015 (WHMIS 2015) for products intended for workplace use.

<sup>2</sup> Testing conducted by Health Canada’s Environmental Health Science and Research Bureau

<sup>3</sup> Testing conducted by Environment and Climate Change Canada’s Ecotoxicology and Wildlife Health Division

# Decisions from Domestic and International Jurisdictions

## Domestic

*B. circulans* is not listed as a reportable or notifiable disease of aquatic animals under the Health of Animals Act, the Reportable Disease Regulations or the Health of Animal Regulations by the Canadian Food Inspection Agency (CFIA, 2014). It is not subject to any plant health requirements according to Invasive Alien Species & Domestic Programs under the Plant Protection Act and Regulations and does not require plant protection permit to import (CFIA, personal communication).

*B. circulans* is considered to be a Risk Group 1 organism for humans and terrestrial animals according to the Public Health Agency of Canada (PHAC, personal communication). Risk Group 1 biological agents are defined as those that may be capable of causing human or animal disease, but are unlikely to do so. These biological agents pose low risk to the health of individuals and/or animals and a low risk to public health, livestock and poultry.

## International

*B. circulans* is not listed as a human pathogen in any of the international pathogenic databases compiled by the American Biological Safety Association (ABSA 2014).

*B. circulans* is categorized as Biosafety Level 1 (BSL-1) microorganism according to the U.S. Center of Disease Control and Prevention (CDC). This level is suitable for work involving well-characterized agents not known to consistently cause disease in healthy adult humans, and of minimal potential hazard to laboratory personnel and the environment.



# 1. Hazard Assessment

## 1.1 Characterization of *Bacillus circulans* ATCC 9500

### 1.1.1 Taxonomic identification and strain history

**Binomial name:** *Bacillus circulans*

**Taxonomic designation:**

**Kingdom:** Bacteria

**Phylum:** Firmicutes

**Class:** Bacilli

**Order:** Bacillales

**Family:** Bacillaceae

**Genus:** *Bacillus*

**Species:** *Bacillus circulans* Jordan 1890 (approved Lists 1980)

**DSL Strain** ATCC 9500

**Superseded Names:** *Bacillus aporrhoeus*

### Strain history

*B. circulans* strain ATCC 9500 was isolated by W.H. Fuller (Fuller and Norman 1943). It was originally deposited by N.R. Smith to the American Type Culture Collection (ATCC 2014) and was eventually deposited to the Belgian Co-ordinated Collection of Micro-organisms (as BCCM accession number of LMG 14421) in 1994 (BCCM 2013). *B. circulans* strain ATCC 9500 has been deposited in a number of other culture collections, as listed in Table 1-1.

**Table 1-1: Listing of current strain designations for *B. circulans* strain ATCC 9500**

<b>Culture Collection</b>	<b>Strain Designations</b>
Bioresource Collection and Research Center, Food Industry Research and Development Institute, Taiwan	BCRC 11721
Korean Collection for Type Cultures, Genetic Resources Center, Korea Research Institute of Bioscience and Biotechnology, Taejon, Republic of Korea	KCTC 1662

Culture Collection	Strain Designations
Belgian Co-ordinated Collections of Micro-organism-organisms (BCCM/LMG), Gent, Belgium	LMG 14421
Biological Resource Center, National Institute of Technology and Evaluation, Chiba, Japan	NBRC 13640
National Collection of Industrial Micro-organisms, Pune, India	NCIM 2234
National Collection of Industrial, Food and Marine Bacteria, Aberdeen, Scotland	NCIMB 9555
Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, US Department of Agriculture, Peoria, IL, USA	NRRL NRS-1108

### 1.1.1.1 Phenotypic and molecular characteristics

The genus *Bacillus* is large, consisting of 11 phylogenetic sub-clusters and over 140 species (Mikkola et al. 2000). The broad range of niches exploited by the genus reflects the wide variation in lifestyles and physiological adaptations among *Bacillus* species (Murray et al. 1995).

*B. circulans* vegetative cells are Gram-positive, motile, straight, round-ended, occasionally slightly tapered and curved, rods with a diameter of 0.6–0.8 µm. They appear singly or in pairs and occasionally short chains. Endospores are ellipsoidal and lie terminally or subterminally in swollen sporangia (Logan and de Vos 2009). Spore dimensions are 0.5–0.7 µm by 4–5 µm (Serraino et al. 2011). Parasporal crystals, such as those observed in *Bacillus thuringiensis*, have not been reported in *B. circulans* (Logan and de Vos 2009).

The name ‘*circulans*’ arose from the observation, made under low magnification, that the interior of colonies of the original isolate flowed in a circular pattern (reviewed in Nakamura and Swezey 1983a); however, only 13% of 61 strains of *B. circulans* examined showed rotating and migrating micro-colonies with spreading growth (Logan et al. 1985). Most strains with motile micro-colonies and spreading growth have since been reassigned to certain *Paenibacillus* species (reviewed in Gillespie and Hawkey 2006). The ability of certain strains to form motile colonies with or without rotating patterns also depends on external factors such as agar and nutrient concentrations. *B. circulans* colonies may also appear in knotted-branching patterns, but only when grown on hard agar surfaces (Komoto et al. 2003).

For *B. circulans* strain ATCC 9500, the ATCC analysis report describes low convex colonies with irregular margins and a finely granular to opaque infrastructure on nutrient agar at 30°C. Growth in broth is slightly turbid with moderate, viscid sediment (ATCC, 2014). In testing at Health Canada, colonies were described as light grey, translucent, round entire and flat on tryptic soy agar at 37°C. Colony sizes are reported in Appendix A, Table A-2.

*B. circulans* is capable of producing acid without gas from a very wide range of carbohydrates except D-arabinose, dulcitol, erythritol, D-fucose, L-fucose, L-sorbose, D-

tagatose and L-xylose (Logan and de Vos 2009). This characteristic can be used to differentiate *B. circulans* from known human and animal pathogens of the *Bacillus cereus* group (*Bacillus anthracis*, *B. cereus* and the insect pathogen *B. thuringiensis* (Rasko et al. 2005)), as shown in Table 1-2.

**Table 1-2: Differential acid production to distinguish between *B. circulans* and three pathogenic *Bacillus* species (Adapted from Logan and de Vos 2009)**

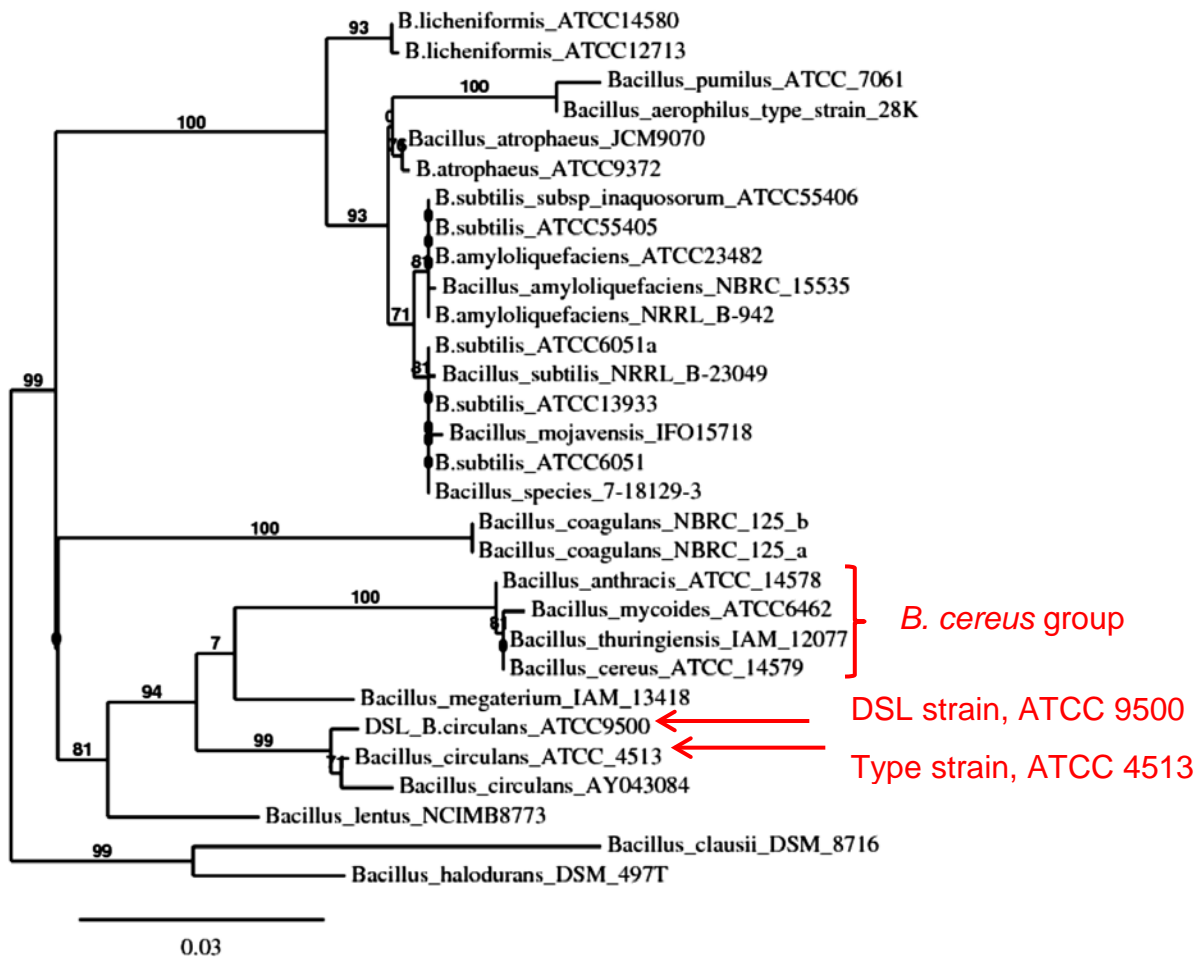
Substrate	<i>B. circulans</i>	<i>B. anthracis</i>	<i>B. cereus</i>	<i>B. thuringiensis</i>
L-Arabinose	+	-	-	-
Galactose	+	-	-	-
Inulin	+	-	-	-
Lactose	+	-	-	-
Melezitose	+	-	-	+
Melibiose	+	-	-	-
Raffinose	+	-	-	-
Glycerol	+	-	+	+
D-Xylose	+	-	-	-
Xylitol	+	-	-	-
Methyl $\alpha$ -D-mannoside	+	-	-	-
Methyl $\beta$ -D-xyloside	+	-	-	-
meso-Inositol	+	-	-	-
D-Mannitol	+	-	-	-

+, > 85% positive strains; -, 0–15% positive strains

The type strain of *B. circulans* (ATCC 4513) contains anteiso-C<sub>15:0</sub> acid as its major cellular fatty acid (57.3%), along with anteiso-C<sub>15:0 iso</sub> and anteiso-C<sub>17:0</sub> (10.0% and 3.4% respectively) (Shida et al.1997). Analysis of *B. circulans* strain ATCC 9500, by Health Canada scientists indicates that the fatty acid profile is comparable with the type strain (as reported in Shida et al.1997), in that anteiso-C<sub>15:0</sub> is the major cellular fatty acid (36.7 - 46.22%) followed by anteiso C<sub>15:0 iso</sub> and anteiso C<sub>17:0</sub> (13.0 -13.8% and 2.3-3.0%, respectively). Phylogenetic analysis by Health Canada scientists based on fatty acid methyl esters using environmental and clinical MIDI databases showed that the DSL strain clusters more closely with a diverse group of non-pathogenic micro-organisms including *Bacillus circulans* - GC subgroup A (see Appendix B, Figure B-1, Figure B-2 and Figure B-3). It also shows that *B. circulans* clusters distantly from bacteria of clinical significance such as *B. anthracis*, *B. cereus*, and *Kocuria* species (see Appendix B, Figure B-2 and Figure B-3).

Phylogenetic analysis performed by Health Canada scientists based on 16S rRNA gene sequences (Figure 1-1) clearly demonstrates that *B. circulans* clusters distantly from known human and animal pathogens (*B. anthracis*, *B. cereus* and the insect pathogen *B. thuringiensis*). It also shows that the DSL strain, ATCC 9500, and the *B. circulans* type strain, ATCC 4513, are closely related. Similarly, the 16S rRNA gene sequence of

the DSL strain shows highest homology with the 16S rRNA gene sequence of the type strain when compared to sequences in the Microseq® full gene library v2.0 (Appendix C, Table C-1).



**Figure 1-1: Phylogenetic relationship of *Bacillus* species based on the alignment of the 16S ribosomal RNA gene sequence coding region**

For many years, *B. circulans* was a poorly defined species originally referred to as a complex rather than species, with members exhibiting phenotypic heterogeneity (Logan and de Vos, 2009). Nakamura and Swezey (1983a) identified at least ten DNA homology groups within this taxon holding non-identical biochemical profiles, suggesting that the phenotypic heterogeneity of the species *B. circulans* was not due to inherent variability of genetically related strains, but to variability introduced by the inclusion of genetically unrelated micro-organisms in that species (Nakamura and Swezey 1983a; Nakamura and Swezey 1983b).

The DSL strain ATCC 9500 is one of nine strains that share a percent guanine cytosine (G+C mol %) range of 38.1-39 mol% with the type strain, ATCC 4513. Only a small number of the 123 strains tested (i.e., 10 out of 18 with G+C mol% of 37 to 41 mol%),

including ATCC 9500, were considered to be closely related to each other and to the type strain, and displayed a minimum amount of phenotypic variation (Nakamura and Swezey 1983a).

On the basis of fatty acid methyl ester and 16S rRNA phylogenetic analysis and G+C mol%, the type strain is considered to be suitable as a surrogate when strain-specific data are not available for the DSL strain; however, it is recognized that closely related strains may acquire different characteristics through lateral gene transfer.

## **1.1.2 Biological and Ecological Properties**

### **1.1.2 Natural occurrence**

*B. circulans* has been isolated from both aquatic and terrestrial environments and in association with animals and plants. It was found in marine water (Das et al. 2008), hot springs (Boulenouar et al. 2006), oil shale site (Dragutinovic' et al. 2012), soil, sewage and the insect gut (Mukhopadhyay et al. 2012). It was also isolated from the fish gut (Bairagi et al. 2004; Kaynar and Beyatli 2009; Saha et al. 2006), gut contents of larvae and adult honey bees, and in honey samples, as part of the normal microbiota from apiarian sources (Gilliam 1997; Alippi et al. 2006; Reynaldi et al. 2004), fresh ark shell (Su et al. 2010), grass (Logan et al. 1985) and the rhizosphere of plants (Mehta et al. 2010).

### **1.1.3 Growth conditions**

*B. circulans* is a chemoorganoheterotroph that uses mono-, di- and polysaccharides and polyhydroxylic alcohols as sources of carbon, energy and electrons (Dragutinovic et al. 2012). It is facultatively anaerobic.

The type strain grows within the temperature range of 14°C-45°C, the pH range of 4.0-10.0 (optimal 7.0) and at 8% sodium chloride (NaCl) (Seiler et al. 2013). ATCC suggests culturing *B. circulans* strain ATCC 9500 at 30°C (ATCC 2014). In testing by Health Canada scientists, *B. circulans* strain ATCC 9500 grew within the tested temperature range of 28°C-42°C in tryptic soy broth (TSB) and on tryptic soy agar, but not in serum-supplemented liquid media that are more representative of a mammalian host environment. In TSB, its optimal growth temperature was 37°C (see Appendix A, Table A-1). The DSL strain was also cultured in tryptic soy agar (see Appendix A, Table A-2).

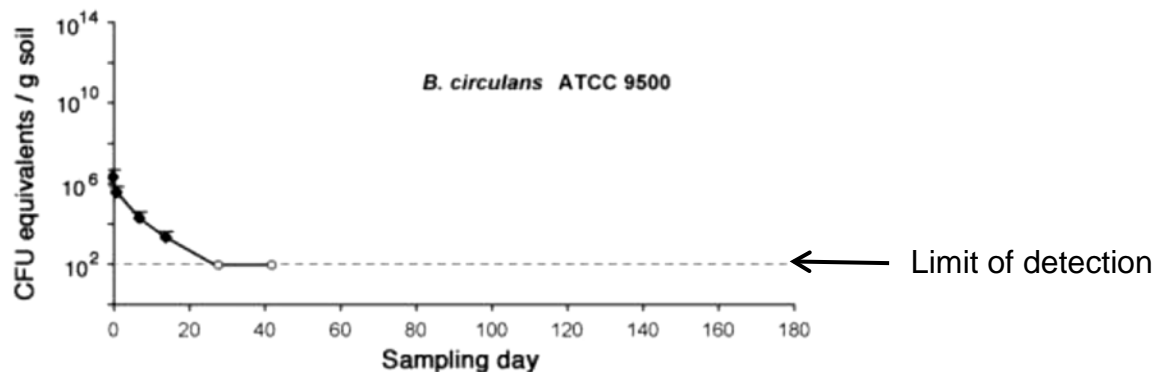
The ability of vegetative cells to grow over a wide range of temperatures, pH and on 8% salt concentrations, and on a variety of substrates allows *B. circulans* to exploit diverse niches.

### 1.1.4 Spore formation

Similar to other *Bacillus* spores, spores of *B. circulans* can resist harsh physical and chemical environmental conditions, such as heat, including the standard sterilization conditions of 121°C for 20 min (Leifert et al. 1989; Trick and Lingens 1985), cold, desiccation, radiation, disinfectants, antibiotics and other toxic agents. As a result they demonstrate extraordinary longevity which makes them ubiquitous and persistent in a variety of different environments (Logan and de Vos 2009). The spores remain in a dormant state for long periods and germinate when conditions are suitable for vegetative growth.

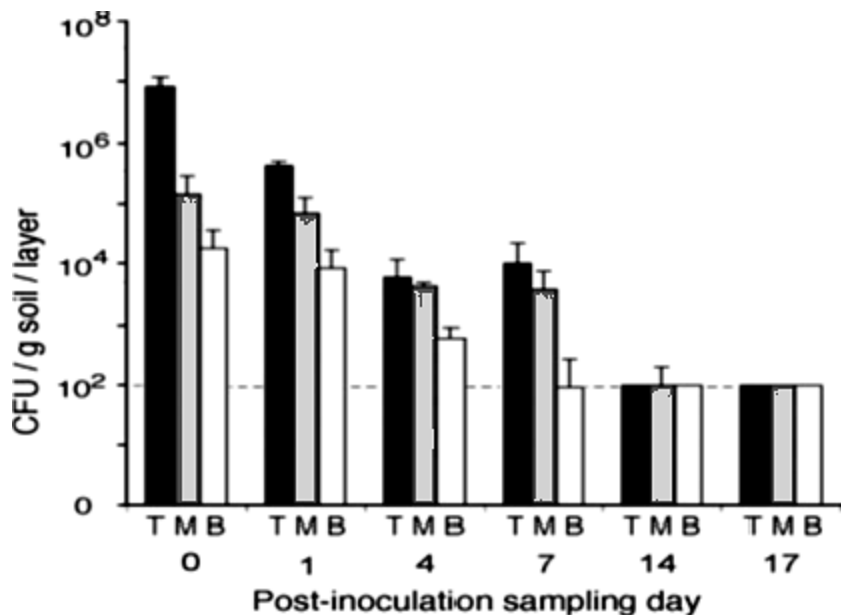
### 1.1.5 Survival and persistence in the environment

In a soil persistence study, vegetative cells of *B. circulans* strain ATCC 9500 could be discriminated from other micro-organisms in an intact soil core microcosm and were quantified using quantitative PCR, targeting strain-specific non-coding regions in the genome identified from amplified fragment length polymorphisms (AFLP). In the top 3 cm of sandy loam soil (pH 5.0, 22°C and 80% relative humidity) inoculated at initial densities of  $\sim 1 \times 10^6$  CFU/g soil, *B. circulans* strain ATCC 9500 declined by day 25 to concentrations near or below the detection limit of  $\sim 1 \times 10^2$  CFU/g soil (Figure 1-2). In a second experiment *B. circulans* strain ATCC 9500 fell below the detection limit by day 14 post inoculation (data not shown) (Providenti et al. 2009).



**Figure 1-2: Persistence of *B. circulans* strain ATCC 9500 in soil, based on qPCR analysis of extractable soil DNA (Can. J. Microbiol., 55, 1166-1175 with permission)**

Another experiment was performed to confirm that the observed rapid decline was not attributable to vertical transport of *B. circulans* strain ATCC 9500 to deeper soil layers. Quantitative PCR applied to 5 cm soil layers from the top (T), middle (M) and bottom (B) of soil cores demonstrated limited vertical dispersal of cells inoculated at the surface of a set of soil cores (Figure 1-3). Samples were taken over time, and the qualitative detection limit of  $\sim 1 \times 10^2$  CFU per gram of soil is represented as a broken line (Providenti et al. 2009).



**Figure 1-3: Vertical transport study in soil-core microcosms, using *B. circulans* strain ATCC 9500 (Can. J. Microbiol., 55, 1166-1175, with permission)**

From inoculation to day 7, *B. circulans* strain ATCC 9500 was detected predominantly in the top layer (98% of total cells on day 0 and 73% of total cells on day 7). Cells were detected in the middle and bottom layers (27% and <0.6% of total, respectively) by day 7. By day 14 post inoculation, cells were only detected in the middle layer at concentrations close to the limit of detection. By day 17, cell numbers dropped below the detection limit in all layers. The authors concluded that the trend of decreasing cell numbers observed in the soil persistence studies was more likely due to death of bacteria than to their transport to deeper layers of the soil core. The authors stated that reports of long term viability of *Bacillus* spores are associated with dry soil conditions. As such, they attributed the limited viability of *B. circulans* strain ATCC 9500 vegetative cells in these studies to the hydration of the soil cores, which were watered every 2-3 days.

In general, introduced microbial populations gradually decline, regardless of the source of their original isolation, due to the hostility of biotic and abiotic conditions in the soil environment (Van Veen et al. 1997). Biotic factors include predation and antagonism; abiotic factors include adverse soil pH, temperature and moisture, and nutrient scarcity (Van Veen et al. 1997). High numbers of vegetative cells are unlikely to be maintained in water or soil due to competition from other microflora (Leung et al. 1995).

### 1.1.6 Antibiotic resistance

Variable antibiotic susceptibility profiles have been reported as part of case reports of infection with *B. circulans* (see section 1.1.3.2).

Susceptibility of *B. circulans* to antibiotics of several classes is strain specific. This applies to the following:

- the beta-lactam antibiotics ampicillin and piperacillin (alone or in combination with tazobactam) (Banerjee et al. 1988; Castagnola et al. 1997; Gurol et al. 2007);
- cefazolin, a first generation cephalosporin (Alebouyeh et al. 2011; Fontana et al. 1997);
- clindamycin (Gurol et al. 2007); and
- vancomycin (Banerjee et al. 1988; Berry et al. 2004; Castagnola et al. 1997; Goudswaard et al. 1995; Krause et al. 1999; Gurol et al. 2007).

*B. circulans* VR0709, a clinical strain resistant to vancomycin was shown to carry the *vanA* gene. In enterococci, *vanA* is generally carried on a transposon (Fontana et al. 1997), but in *B. circulans* it is located on the chromosome (Ligozzi et al. 1998) and as such may not be readily transferable to other micro-organisms. No other reports of *vanA* or vancomycin resistance in *B. circulans* were identified in the scientific literature. Given that *B. circulans* is only rarely a pathogen and it is susceptible to a number of antibiotics (Fontana et al. 1997), its resistance to vancomycin does not represent a serious therapeutic challenge (Fontana et al. 1997; Krčméry Jr and Sefton 2000).

Like many micro-organisms, *B. circulans* is resistant to antibiotics it produces, and those of the same class. Certain strains of *B. circulans* produce biturosin, a member of the neomycin family of aminoglycosides antibiotics, and are resistant to a variety of aminoglycosides through the activity of an aminoglycoside phosphotransferase (APH). Similarly, as a circulin-producing organism, *B. circulans* is resistant to both circulin and the related antibiotic polymyxin (Storm et al. 1977).

*B. circulans* strain ATCC 9500 was tested against antibiotics from a number of families by scientists at Health Canada (Table 1-3).

**Table 1-3: Minimum Inhibitory Concentrations (MIC, µg/mL) of vegetative cells of *B. circulans* strain ATCC 9500**

Antibiotic	MIC (µg/mL) <sup>a</sup>	Susceptible <sup>b</sup>	Intermediate <sup>b</sup>	Resistant <sup>b</sup>	MIC Interpretation
Amoxicillin	0.37	N/A	N/A	N/A	N/A
Cephotaxime	3	≤8	16-32	≥64	Susceptible
Ciprofloxacin	0.37	≤1	2	≥4	Susceptible
Doxycycline	0.37	N/A	N/A	N/A	N/A
Erythromycin	3	≤0.5	1-4	≥8 (≥4 <sup>c</sup> )	Intermediate
Gentamicin	0.37	≤4	8	≥16 (≥4 <sup>c</sup> )	Susceptible
Meropenem	0.37	N/A	N/A	N/A	N/A
Nalidixic acid	12	N/A	N/A	N/A	N/A
Trimethoprim	12	≤2	N/A	≥4	Resistant
Vancomycin	0.37	≤4	N/A	N/A (≥4 <sup>c</sup> )	Susceptible

N/A, not available



<sup>a</sup> TSB-MTT liquid assay method was used to determine the MIC values (Seligy and Rancourt, 1999). Values correspond to the minimal inhibitory concentration ( $\mu\text{g/mL}$ ) for *B. circulans* strain ATCC 9500 grown in the presence of antibiotic for 24 hours at 37°C

<sup>b</sup> Interpretive criteria (MIC  $\mu\text{g/mL}$ ; CLSI 2010)

<sup>c</sup> Interpretive criteria (MIC  $\mu\text{g/mL}$ ; EFSA 2008)

### 1.1.2.6 Pathogenic and toxigenic characteristics

In order to be an effective microbial pathogen, a micro-organism must be able to adhere to host cell surfaces, invade host tissues and evade host defences. In one study, certain *B. circulans* isolates had some ability to adhere or invade Hep-2 and Caco-2 cell lines while others were completely incapable of adherence or invasion (Rowan et al. 2001).

Cytotoxic activity refers to the destructive action of a micro-organism or its products which cause animal cells to undergo structural and metabolic damage, could facilitate invasion and are associated with virulence (Rowan et al. 2001; Kim et al. 1998). Cell-free culture supernatants of two clinical and one food isolate of *B. circulans* implicated in food poisoning had cytotoxic activity towards both human Caco-2 and HEp-2 epithelial cell lines (Rowan et al. 2001). Analysis by Health Canada scientists testing vegetative cells of *B. circulans* strain ATCC 9500 against two different cell lines (human colonic and murine macrophages), showed no significant cytotoxic activity.

Isolates of *B. circulans* exhibit varying levels of hemolysis (Rowan et al. 2001). For all enterotoxigenic strains, discontinuous beta hemolysis was apparent following incubation on sheep blood agar plates (Phelps and McKillip 2002). However, analysis on the DSL strain of *B. circulans*, by Health Canada scientists, indicated no hemolytic activity.

Lecithinase is a bacterial enzyme that increases virulence by destabilizing the host cell membranes (Todar 2012). Phelps and McKillip (2002) showed lecithinase production on egg yolk-polymyxin B agar for all *B. circulans* strains tested, suggesting that it is also likely to be produced by the DSL strain ATCC 9500.

Food-poisoning strains of *B. circulans* have been reported to produce toxins (Beattie and Williams 1999), including some that are similar to the *B. cereus* hemolysin BL (HBL) enterotoxin (Rowan et al. 2001) and non-hemolytic enterotoxin (Nhe) (Phelps and McKillip 2002). Using PCR, the BceT diarrheal toxin and Nhe genes were identified in food isolates (Phelps and McKillip 2002) and the HBL toxin complex was identified only in clinical isolates of *B. circulans* (Rowan et al 2001; Phelps and McKillip 2002). The latter results were also in agreement with detection of secreted HBL enterotoxin in culture supernatants of one of the clinical strains, using a commercial reversed passive latex agglutination assay (RPLA) (Rowan et al. 2001). In Health Canada's independent analysis, *B. circulans* strain ATCC 9500 was negative for both HBL and Nhe toxins using the Duopath™ *Cereus* Enterotoxin kit (Merck Millipore) and negative for the *Bacillus* diarrhoeal enterotoxin using the 3M™ Tecra™ Pathogen and Toxin visual immunoassay.

### 1.1.2.7 Horizontal gene transfer

Like other bacteria, *B. circulans* strain ATCC 9500 can acquire virulence genes through horizontal gene transfer. However, with the exception of a single report of *B. circulans* harboring one of the two *B. anthracis* virulence plasmids (Luna et al. 2006), lateral transfer of virulence determinants has not been reported in *B. circulans*. In independent testing by Health Canada scientists, *B. anthracis* plasmids were not detected in ATCC 9500.

With the likely exception of lecithinase, *B. circulans* strain ATCC 9500 does not appear to possess virulence genes that could be transferred to other bacteria in the environment. As lecithinase is already widely present, the potential lateral transfer of lecithinase activity from ATCC 9500 to other bacteria is not considered to represent an additional hazard.

### 1.1.7 Effects

#### 1.1.3.1 Environment

##### Plants

*B. circulans* was isolated from the rhizosphere soil of *Halophila ovalis* a type of seagrass, apparently as part of the normal microbiota, where its phosphate solubilizing properties may benefit the plant by providing a nutritional advantage (Ghosh et al. 2012). A comprehensive scientific literature search did not reveal any further reports of *B. circulans* in association with aquatic plants.

*B. circulans* can be beneficial to terrestrial plants because it shows antimicrobial activity towards plant pathogenic fungi through the activity of glucanolytic, chitinolytic, and proteolytic enzymes. Affected fungi include *Verticillium dahliae*, *Phytophthora cactorum*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum* (Berg et al. 2002). *B. circulans* also inhibits mycelial growth in *Fusarium oxysporum*. Treatment of sorghum seedlings with *B. circulans* ( $3 \times 10^9$  CFU per pot), resulted in 95.83% *F. oxysporum* root and crown rot suppression in 4 week old sorghum plants (Idris et al. 2007). *B. circulans* WL-12 is capable of secreting multiple chitinases (A1, A2, B1, B2, C and D), (reviewed in Mustafa A Alam et al. 1995), and when grown in a medium containing cell walls of *Pyricularia oryzae* (the rice blast disease pathogen), *B. circulans* WL-12 produced  $\beta$ -1,3-glucanase,  $\beta$ -1,6-glucanases and chitinase that caused selective lysis of *P. oryzae* cell walls (Tanaka and Watanabe 1995). Another strain of *B. circulans* was also shown to produce chitinases, had antibacterial activity against both Gram positive and Gram negative bacteria and demonstrated some antifungal activity against the unicellular fungi (MIC value in range of 0.5-2 $\mu$ g/mL, against all organisms tested) (Abada et al. 2014).

*B. circulans* also produces a xylanase (BcX) with potential hypersensitizing properties in plants; however, in vitro treatment of suspension-cultured tobacco (*Nicotiana tabacum* cv. Xanthi) cells with BcX did not induce any hypersensitivity in them (Yano et al. 1998).

In a greenhouse study, *B. circulans* culture filtrate was shown to make a 40.7% reduction in the mean number of lesions caused by the Tomato mosaic tobamovirus (ToMV) on the leaves of infected tomato plants (*Lycopersicon esculentum*) (Megahed et al. 2013).

Plant growth promotion by *B. circulans* has been shown in the following studies:

- Inoculation of planted seeds of red clover (*Trifolium pretense*) with *B. circulans* at a concentration of  $3.44 \times 10^{10}$  CFU per pot, produced no negative effects on seedling emergence or on length or dry mass of shoots or roots. Instead, root lengths and shoot masses of *B. circulans*-treated plants were significantly higher than controls (Chitty 2005).
- *B. circulans* MTCC 8983 efficiently solubilized inorganic phosphate in Pikovskaya broth, producing 957.3 mg/L soluble phosphate within 72 hours in vitro. It also produced plant protecting substances such as indole acetic acid (up to 15.13  $\mu\text{g/mL}$ ) and siderophores, and inhibited growth of the fungal plant pathogen *Dematophora necatrix* by 46.57%. (Mehta et al. 2010).
- In a net house setting, 60 days after inoculation of tomato seeds with *B. circulans* CB7, a remarkable increase was observed in seed germination (22.32%), shoot length (15.91%), root length (25.10%), shoot dry weight (52.92%) and root dry weight (31.4%), as well as nitrogen (18.75%), potassium (57.69%), and phosphorus (22.22%) content of shoot biomass over controls. Significantly higher levels of nitrogen, phosphorus and potassium were also observed in the soil of treated plants as compared to untreated controls. Phosphate solubilisation, production of auxin, 1-aminocyclopropane-1-carboxylate deaminase, siderophores, nitrogenase activity, and antagonism of *D. necatrix* contributed to plant growth promotion (Mehta et al. 2014).
- Inoculation of wheat (*Triticum aestivum* L.) with *B. circulans*, significantly enhanced root colonization by the vesicular-arbuscular mycorrhizal fungus (VAM), yield of grain and straw (2.261 gram/pot vs. 2.060 g/pot), and uptake of nitrogen (1.720 mg/pot vs. 1.330 mg/pot) and phosphorus (0.471 mg/pot vs. 0.371 mg/pot) compared with untreated controls (Singh and Kapoor 1999).

As a contaminant, *B. circulans* can have detrimental effects on plant tissue cultures (Leary et al. 1986; Trick and Lingens 1985). Tissue culture and greenhouse studies suggest that *B. circulans* may be pathogenic to date palms: *B. circulans* isolated from healthy heart tissue, vegetative bud meristem, shoot primordia, young branch bract, and mature fronds was used to inoculate healthy offshoots of date palms of different cultivars and caused adverse effects. At  $10^5$  CFU per palm callus *B. circulans* produced necrosis and a slimy and destructive soft rot in tissue cultures (Leary et al. 1986)). At  $10^6$  CFU per plant, the majority of greenhouse seedlings showed necrosis that progressed down the cotyledon, followed by wilting (greenhouse study, Leary and Chun (1989)).

Given the heterogeneity that exists within the species *B. circulans*, it is unclear to what extent the properties observed in other *B. circulans* strains are shared by the DSL strain

of *B. circulans*; however, consideration of all effects attributed to the species provides an understanding of the spectrum of characteristics that could be attributed to the DSL strain (both beneficial and harmful).

## Invertebrates

Aquatic invertebrates *Daphnia magna* and *Artemia* species have been successfully used as live vectors for the probiotic delivery of *B. circulans* to fish in a process termed bioencapsulation (Faramarzi et al. 2012a; Faramarzi et al. 2012b; Sahandi et al. 2012). No adverse effects on the bioencapsulated invertebrates were reported for the duration of the studies (up to four weeks).

*B. circulans* isolated from the larva of the southern house mosquito (*Culex quinquefasciatus*) killed larvae of three medically important mosquito species. Compared to a highly virulent entomopathogen (*B. sphaericus* strain 2362), *B. circulans* was less toxic to *C. quinquefasciatus* (median lethal concentration [LC<sub>50</sub>] (854 vs. 17947 spores/mL), and the anopheles mosquito (*Anopheles gambiae*) (LC<sub>50</sub>: 2 268 vs. 14 447 spores/mL). However, when tested against the yellow fever mosquito (*Aedes aegypti*), *B. circulans* was 107 times more toxic than *B. sphaericus* strain 2362 (LC<sub>50</sub> 13,739 vs.  $1.47 \times 10^6$  spores/mL) and as pathogenic as another larvicidal pathogen *B. thuringiensis* ssp. *israelensis* (data not shown) (Darriet and Hougard 2002).

(Singer et al. 1997) also studied toxicological effects of *B. circulans* on aquatic invertebrates. In spite of its methodological shortcomings, the study clearly showed strong molluscicidal activity against the D-stage veliger of the zebra mussel (*Dreissena polymorpha*) for *B. circulans* strain 42G1 when compared to four other *Bacillus* species including two strains of *Bacillus alvei*. In the same study *B. circulans* showed considerably lower biological activity against the adult fresh water snails (*Biomphalaria glabrata*), which was comparable to the activities of two strains of *B. alvei* and the single strain of *Bacillus brevis* tested.

A comprehensive scientific literature search did not reveal any further reports of the DSL strain or other strains of *B. circulans* in association with aquatic invertebrates.

Adverse effects of *B. circulans* in terrestrial invertebrates have been identified in the context of biocontrol studies:

- Experimental challenge of third instar larvae of the click beetle (*Agriotes lineatus*) with potato dipped in a suspension of  $1.8 \times 10^9$  CFU/mL *B. circulans* strain Ar1 as a food source resulted in 100% mortality within ten days (Danismazoglu et al. 2012). Another strain of *B. circulans* was found to cause mortality most frequently in the fourth instar larvae of the snout moth (*Locastra muscosalis*) (Sharma et al. 2006).
- *B. circulans* Ar1 applied to the adult Hazelnut Leaf Holer (*Anoplus roboris*) produced 33% mortality within eight days (Demir et al. 2002).

- Nematicidal activity was observed in a greenhouse experiment with *Meloidogyne incognita*-infested tomato plants (previously inoculated with 1000 fresh J2 nematodes per pot). *B. circulans* KSB2 applied at  $2 \times 10^8$  CFU per pot significantly reduced nematode populations: 67.4% fewer hatched juveniles/root, 57.1% fewer females per root and 79.3% fewer juveniles per kg soil were observed compared to negative controls, 30 days after *B. circulans* was applied. Further reductions of 41.3%, 41.25% and 57.8% respectively, were observed at 60 days following inoculation (El-Hadad et al. 2011).
- Nematicidal activity was also observed against the parasitic nematode of sheep, *Haemonchus contortus*. Inoculation of infested sheep feces with approximately  $2 \times 10^8$  CFU/mL *B. circulans* caused a significant reduction in the number of larvae in the treated feces (80.9%,  $p > 0.05$ ) as compared to the negative control (Sinott et al. 2012). Similarly, in a different study, oral administration of a spore suspension of *B. circulans* to lambs infected with *H. contortus*, at  $2 \times 10^9$  CFU /day for a period of 5 days caused a significant reduction (~87%,  $p < 0.05$ ) in larval development in the treated animals (Sinott et al. 2014).

*B. circulans* may be beneficial to honey bees. It was isolated from honey (Alippi and Reynaldi 2004; Reynaldi et al. 2004), the pollen collected from the legs (Gilliam 1979) and gut of healthy worker honey bees (reviewed in Gilliam 1979 and Gilliam 1997), where it may play a role in the production and preservation of bees' pollen. It also inhibits mycelial growth of *Ascosphaera apis*, the fungus that causes chalkbrood disease in honey bee larvae (reviewed in Reynaldi et al. 2004). Other bee species may indirectly be adversely affected by *B. circulans*. It was suggested to be harmful to alfalfa leaf cutting bee (*Megachile rotundata*) populations due to its interference with larval development by spoiling nest cell provisions (Goerzen 1991).

Environment and Climate Change Canada scientists investigated effects in springtails. Adult springtails (*Folsomia candida*) were experimentally challenged with the DSL strain (28 days incubation with  $2.45 \times 10^9$  CFU *B. circulans* strain ATCC 9500 per test jar). This challenge did not cause any significant reductions in the number of adults that survived and in the total amount of juveniles produced in each test unit, as compared to the negative controls (Chitty 2005).

## **Vertebrates**

There are several reports of isolation of *B. circulans* as part of the normal flora of the digestive tract of different species of fish such as rohu, (from the abstract of Ghosh et al. 2002), tilapia and grass carp (unidentified strains) (Saha et al. 2006), where it may have a probiotic effect, as observed in the following feeding studies:

- Supplementation of feed with *B. circulans* strain PB7 (isolated from *Catla catla* intestine) at  $2 \times 10^5$  cells per 100 g feed, caused significantly superior growth performance in fingerlings of *C. catla* in terms of live weight gain, specific growth rate and protein efficiency ratio). Fish fed *B. circulans*-supplemented feed survived challenge with the fish pathogen (*Aeromonas hydrophila*) significantly

better than controls (96.66% vs. 6.66% survival) (Bandyopadhyay and Das Mohapatra 2009).

- Feeding a probiotic formulation (including *B. circulans* and two other bacilli) bioencapsulated in *D. magna*, to Persian sturgeon larvae (*Acipenser persicus*) significantly reduced ammonia and urea excretion and increased protein retention, and enhanced resistance to stressors including extremes of pH, salinity, temperature and ammonia. Compared to untreated controls, a significantly higher percentage of treated fish survived (Faramarzi et al. 2012b).
- Other studies showed significant positive effects in common carp (*Cyprinus carpio*) (Jafaryan et al. 2011), rainbow trout (*Oncorhynchus mykiss*) (Jafarian et al. 2009), silver carp (*Hypophthalmichthys molitrix*) (Adineh et al. 2011) and in grass carp (*Ctenopharyngodon idella*) (Sahandi et al. 2012). No associated adverse effects were reported as a result of consumption of the probiotic formulations in any of the studies reviewed.

A comprehensive scientific literature search did not reveal any further reports of the DSL strain or other strains of *B. circulans* in association with aquatic vertebrates.

In an oral administration study, mice (ICR, male and female, n = 5/sex) were given a single inoculation of either the culture medium (control group) or live *B. circulans* (strain unknown;  $7.0 \times 10^8$  CFU/animal). There were no deaths, clinical signs or reductions in body weight during the 14-day observation period (reviewed in FSANZ).

Given the heterogeneity that exists within the species *B. circulans*, it is unclear to what extent the properties observed in other *B. circulans* strains are shared by *B. circulans* strain ATCC 9500; however, consideration of all effects attributed to the species provides an understanding of the spectrum of characteristics that could be attributed to the DSL strain (both beneficial and harmful).

### 1.1.3.2 Humans

With the exception of *B. cereus*, *Bacillus* infections in humans are rare. They are diverse and tend to occur in immunocompromised people (Pennington et al. 1976), or in association with implanted medical devices (Banerjee et al. 1988) or recent trauma (Logan et al. 1985). There are few reported cases of *B. circulans* infection, most of which were responsive to antimicrobial therapy:

- *B. circulans* bacteremia has been reported in cancer patients with multiple medical problems (Banerjee et al. 1988);
- *B. circulans* RIGLD BC1 was isolated from blood in a case of bacteremia in an immune-compromised individual with end stage renal disease. Despite antimicrobial therapy, the patient died (Alebouyeh et al. 2011).
- *B. circulans* was indicated as the possible cause of infection in a case of fatal meningitis in a child (Boyette and Rights 1952). However, identification of *B. circulans* as the infectious agent was not fully substantiated.

- *B. circulans* was implicated as the causative agent in infections involving presence of indwelling catheters (Berry et al. 2004; Fontana et al. 1997; Roncoroni et al.1985). There are also other instances in which usage of catheters were suspected as the point of introduction of *B. circulans* into the patients organs such as in a case of cholecystitis (Khatib et al. 1995) and in a case of paracardiac infection post stem cell transplantation (Gurol et al. 2007). Intraoperative contamination involving transplantation of a mechanical heart valve has been blamed for a case of endocarditis in a patient with a history of aortic valve replacement (Krause et al. 1999).
- *B. circulans* has been isolated as the putative agent in wound infections in the case of a patient with malignant ovarian carcinoma following a total abdominal hysterectomy (Logan et al.1985), in a case of finger cellulitis in an otherwise healthy individual following a human bite (Goudswaard et al. 1995) and in a case of endocarditis involving an immunocompromised individual (Gatermann et al. 1991).
- *B. circulans* was isolated in at least 13 cases of endophthalmitis following cataract surgery or intraocular lens implantation in a hospital in Canada in 1993 (CDC 1996). In most of the reported cases, intraoperative use of contaminated solutions was blamed for the infections. The majority of all those cases led to vitrectomy.

Given the heterogeneity that exists within the species *B. circulans*, it is unclear to what extent the properties observed in other *B. circulans* strains are shared by *B. circulans* strain ATCC 9500; however, consideration of all effects attributed to the species, provides an understanding of the spectrum of characteristics that could be attributed to the DSL strain (both beneficial and harmful).

## **1.2 Hazard severity**

### **1.2.1 Environment**

The environmental hazard potential of *B. circulans* strain ATCC 9500 is assessed to be low since there is no evidence to suggest that *B. circulans* is pathogenic to aquatic or terrestrial plants or to vertebrates at the population level in the environment, in spite of its widespread distribution in the environment. Instead, there is evidence to suggest that *B. circulans* has beneficial effects as a probiotic in fish and a plant growth-promoting rhizobacterium.

Information from the scientific literature indicates that other certain strains of *B. circulans* could have pathogenic potential in both aquatic and terrestrial invertebrates. However, the effects seen were mainly under experimental challenge when insects, pest nematodes and molluscs were directly treated with high concentrations of *B. circulans* as part of experimental investigations into its biocontrol potential.

Although there is a lack of specific pathogenicity/toxicity testing for the DSL listed strain

on invertebrates, the overall hazard severity to the environment of *B. circulans* strain ATCC 9500 is estimated to be low given evidence that it does not possess any known virulence characteristics based on the Health Canada scientists in vitro studies and based on the fact that the DSL listed strain has been released into the Canadian environment without any reported adverse effects.

### **1.2.2 Human health**

The human hazard potential of *B. circulans* strain ATCC 9500 is assessed to be low because in spite of its widespread distribution and history of use in consumer and commercial products. There are few reported cases of *B. circulans* infection, and these are limited to individuals who were predisposed to infection because of compromised immunity or debilitating disease and are primarily associated with implantation of contaminated medical devices or failures of aseptic surgical practices. Most cases were responsive to antimicrobial therapy.

No human infections have been specifically attributed to the DSL strain *B. circulans* strain ATCC 9500. In testing by Health Canada scientists, no cytotoxicity or hemolytic activity was observed in vitro with *B. circulans* strain ATCC 9500. In the unlikely event of infection with *B. circulans* strain ATCC 9500, there are effective antibiotic treatments available.

Hazards related to micro-organisms used in the workplace should be classified accordingly under the Workplace Hazardous Materials Information System (WHMIS)<sup>4</sup>.

## **2. Exposure Assessment**

### **2.1 Sources of exposure**

This assessment considers exposure to *B. circulans* strain ATCC 9500 resulting from its addition to consumer or commercial products or its use in industrial processes in Canada.

*B. circulans* strain ATCC 9500 was added to the DSL in 1997 because it was manufactured in or imported into Canada for use in consumer or commercial products between January 1, 1984 and December 31, 1986.

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<sup>4</sup> A determination of whether one or more criteria of section 64 of CEPA are met is based on an assessment of potential risks to the environment and/or human health associated with exposure in the general environment. For humans, this includes, but is not limited to, exposure from air, water and the use of products containing the substances. A conclusion under CEPA may not be relevant to, nor does it preclude, an assessment against the criteria specified in the *Hazardous Products Regulations*, which is part of the regulatory framework for the Workplace Hazardous Materials Information System (WHMIS) for products intended for workplace use.



Responses to a voluntary questionnaire, sent in 2007 to a subset of key biotechnology companies in Canada, combined with information obtained from other federal regulatory and non-regulatory programs, indicate that up to 14650 kg of products potentially containing *B. circulans* strain ATCC 9500 and up to a total of  $1.35 \times 10^{15}$  CFU *B. circulans* strain ATCC 9500 in other products (formulation and concentration unknown in both cases), were imported into or manufactured in Canada in 2006.

The Government conducted a mandatory information-gathering survey under section 71 of CEPA, as published in the Canada Gazette, Part I, on October 3, 2009 (section 71 notice). The section 71 notice applied to any persons who, during the 2008 calendar year, manufactured or imported *B. circulans* strain ATCC 9500, whether alone, in a mixture or in a product. Responses indicated that approximately 153 kg of products containing *B. circulans* strain ATCC 9500 were imported in Canada in 2008 for consumer and commercial uses.

*B. circulans* strain ATCC 9500 has properties that make it of commercial interest for a variety of uses. A search of the public domain identified the following ongoing consumer, commercial and industrial applications of other naturally occurring *B. circulans* strains.

*B. circulans* is primarily used as a production organism for a variety of enzymes (e.g.,  $\beta$ -amylases,  $\beta$ -galactosidase, cellulases, chitinases, cyclodextrin, glucanotransferases, proteases, xylanases and alginase) and specialty chemicals for:

- Food, cosmetic and pharmaceutical industries (EU Scientific Committee, 1997; Katase et al. 2012; Napier 1978; CIPO Patent 1025383; Qi and Zimmermann 2005);
- Aquaculture (Saha et al. 2006);
- Bioremediation of biological waste (Patil and Chaudhari 2013);
- Bleaching of paper pulp, (reviewed in Dhillon and Khanna, 2000; Dhillon et al. 2000);
- Biodegradation of feathers and animal hair (Subba Rao et al. 2009);
- Leather processing (Subba Rao et al. 2009);
- Detergent formulations (Benkiar et al. 2013) and;
- Treatment of sewage and waste water, particularly for the DSL strain (Bianchi et al. 2009).

Despite the interest in *B. circulans* strain ATCC 9500 for the production of food enzymes, to date no enzymes from this source organism have been approved in Canada.

*B. circulans* is also used in the production of antimicrobial compounds (Das et al. 2008; Dion et al. 1972; Epanand and Vogel 1999; Fujikawa et al. 1965; Hayashi et al. 1968; Murao et al. 1974; Sogn 1976).

B. circulans could potentially be used to colonize a particular environment and perform a specific function in situ, such as:

- Degradation of organic compounds, including fluoranthene and chlorinated hydrocarbons (Kafilzadeh et al. 2013);
- Mineral leaching and biosorption of heavy metals (Dragutinovic et al. 2012; Groudev 1987; Khanafari et al. 2008; Pradhan et al. 2006; Yilmaz 2003) and;
- As part of a bacterial preparation for agricultural use (US Patent 5733355).
- For use in performance testing of media, stains, reagents and identification kits, and for the evaluation of bacteriological procedures (Product Sheet A-1, 2014);
- For use as a control organism in diagnostic microbiological procedures (Product Sheet A-2, 2014);
- Waste digester and odour counteractant intended for use in grease traps, drain pipes, lift stations, septic tanks, portable toilets and odorous surfaces (Product Sheet B, 2014);
- Water restoration of ponds (Product Sheet C, 2011) and;
- For use as feed probiotic in aquaculture (Product Sheet D, 2014).

A recent increase in the number of publications related to this organism may reflect a growing commercial interest in B. circulans.

## **2.2 Exposure Characterization**

### **2.2.1 Environment**

The environmental exposure to B. circulans strain ATCC 9500 is expected to be medium based on responses to the section 71 notice.

The magnitude of exposure of B. circulans strain ATCC 9500 to environmental species and the Canadian ecosystem will depend on the nature of the use and on its persistence and survival in the environment to which it is released.

The concentration B. circulans strain ATCC 9500 introduced into soil at  $\sim 1 \times 10^4$  CFU/g was demonstrated to fall to levels near or below the detection limit of  $\sim 1 \times 10^2$  CFU/g soil after only 25 days (Providenti et al. 2009). High numbers of vegetative cells are unlikely to be maintained in water or soil due to competition for nutrients (Leung et al. 1995) and microbiostasis, which is an inhibitory effect of soil, resulting in the rapid decline of populations of introduced bacteria (van Veen et al. 1997).

Spores of B. circulans strain ATCC 9500 are more resistant to harsh conditions compared to their vegetative counterparts. Under dry conditions, spores of B. circulans strain ATCC 9500 are likely to persist and accumulate in the environment.

In the case of aquatic ecosystems, exposure to B. circulans strain ATCC 9500 is expected to be greatest for organisms in the vicinity of direct application such as in water treatment and where wastewater effluents are released subsequent to waste

water treatment drain cleaning or degreasing. The amount of exposure will depend on the mode of application, the volume and concentration of products applied and proximity of aquatic species to the point of introduction.

Similarly, the exposure of terrestrial ecosystems to *B. circulans* strain ATCC 9500 is expected to be greatest in the environment surrounding the sites of direct application such as for biodegradation or bioremediation of contaminants in soil. The amount of exposure will depend on the mode of application, the volume and concentration of products applied and proximity of terrestrial species to the point of introduction. Spores could become airborne from soils recently treated with *B. circulans* strain ATCC 9500 and could be inhaled by environmental species and widen the area in which terrestrial species could be exposed.

Exposure as a result of releases of *B. circulans* strain ATCC 9500 from facilities manufacturing enzymes could occur but is expected to be limited by the application of good manufacturing processes. Exposure of environmental species to the micro-organism via facility release is expected to be limited.

### **2.2.2 Human**

Human exposure to *B. circulans* strain ATCC 9500 is expected to be medium based on the wide range of uses reported in the section 71 survey.

Human exposure is expected to be greatest through the direct use of consumer products containing spores or viable cells of *B. circulans* strain ATCC 9500. Handling and application of such products would be expected to result in direct exposure to aerosolized droplets or airborne spores via dermal or inhalation routes. Inadvertent ingestion following use on or near food preparation surfaces and contact with the eyes, are possible secondary routes of exposure.

Humans may also be exposed as bystanders during commercial application of cleaning, water treatment, agricultural or biodegradation products. The extent of bystander exposure will depend on the mode of application, the volume applied and the proximity of bystanders to the site of application. In general, exposure is expected to be low for these applications.

Indirect exposure to *B. circulans* strain ATCC 9500 in the environment subsequent to its use in water and wastewater treatment, water restoration of closed or slow flow water systems, drain cleaning and degreasing of sewer lines or disposal of waste from its use in the production of enzymes is also likely to occur in the vicinity of application or disposal sites, but is expected to be less than direct exposure from the use of the organism in consumer products.

In the event that *B. circulans* strain ATCC 9500 enters municipal drinking water treatment systems through release from intended and potential uses, drinking water treatment processes (e.g., coagulation, flocculation, ozonation, filtration and

chlorination) are expected to effectively eliminate these micro-organisms, and so limit ingestion.

Release of *B. circulans* strain ATCC 9500 from facilities manufacturing enzymes or biochemicals could occur, but is expected to be limited by the application of good manufacturing practices, in which measures should be taken to minimise the probability of releases of production micro-organisms.

Growth in the market for “greener” microbial-based products may increase human exposure to the DSL *B. circulans* strain which have potential applications in these products.

### **3. Risk Characterization**

In this assessment, risk is characterized according to a paradigm whereby a hazard and exposure to that hazard are both required for there to be a risk. The risk assessment conclusion is based on the hazard, and on what is known about exposure from current uses.

Hazard has been estimated for *B. circulans* strain ATCC 9500 to be overall low for the environment and for human health. Environmental and human exposure to *B. circulans* strain ATCC 9500 is estimated to be medium because imported products were reported in the section 71 survey. Nevertheless based on the low estimation of hazard, the risk associated from this organism from current levels of exposure is estimated to be low.

The determination of risk from current uses is followed by consideration of the estimated hazard in relation to foreseeable future exposures (from new uses).

*B. circulans* strain ATCC 9500 has useful properties that make it of interest for use in additional industrial processes or consumer or commercial products. In the event that these potential consumers, commercial or industrial uses of *B. circulans* strain ATCC 9500 are realized, the level of environmental and human exposure to these strains could increase. Nevertheless, the risk from foreseeable potential uses of *B. circulans* strain ATCC 9500 remains low given that there is no evidence of effects on human health or adverse ecological effects at the population level for environmental species, in spite of the potential industrial, environmental, and commercial uses of *B. circulans* strain ATCC 9500.

### **4. Conclusion**

Based on the information presented in this screening assessment, it is concluded that *B. circulans* strain ATCC 9500 is not entering the environment in a quantity or concentration or under conditions that:

- have or may have an immediate or long-term harmful effect on the environment or its biological diversity;

- constitute or may constitute a danger to the environment on which life depends;  
or
- constitute or may constitute a danger in Canada to human life or health.

Therefore, it is concluded that *B. circulans* strain ATCC 9500 does not meet the criteria set out in section 64 of the CEPA.

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## Appendices

### Appendix A: Growth of *Bacillus circulans* ATCC 9500 in Various Temperatures

**Table A-1: Growth of *B. circulans* strain ATCC 9500 at different temperatures in liquid media after 24 hours**

Media	28°C	32°C	37°C (optimal growth)	42°C
Tryptic Soy Broth (TSB)	+	+	+	~
10% Fetal Bovine Serum (FBS)	x	~	~	x
100% FBS	x	x	x	x
Dulbecco's Modified Eagle's Medium (DMEM) w FBS and Glutamine	x	x	±	x

+ Optical density (OD) > 0.2; ~, OD < 0.2; ±, OD < 0.1; x, OD < 0.05

**Table A-2: Size (in mm) of *B. circulans* strain ATCC 9500 colonies on Tryptic Soy Agar (TSA) plates after 24 hours and 48 hours**

Incubation time	28°C	30°C	32°C	37°C	42°C
24 h	1.5	0.1	1.5	2	2
48 h	3	0.5	2	5	4

## Appendix B: Characteristics of *Bacillus circulans* ATCC 9500- Fatty Acids Methyl Ester (FAME) Analysis

Table B-1: MIDI identification\* of *B. circulans* strain ATCC 9500

Database	Fatty Acid Profile Similarity Index	Best Match
Environment	0.400 (8/8)	Bacillus-circulans-GC subgroup A
Clinical	0.391 (6/9)	Bacillus-circulans-GC subgroup A
Clinical	0.494 (3/9)	Bacillus-megaterium

\* Data generated by Health Canada's Healthy Environments and Consumer Safety Branch shows the best match between the sample and the environmental and clinical MIDI databases and the fatty acid profile similarity index (average of all matches) along with the number of matches (number of matches/total number of tests, parentheses). For methods and additional details, see [www.midilabs.com/fatty-acid-analysis](http://www.midilabs.com/fatty-acid-analysis). As a general rule of thumb, samples that cluster within a Euclidian distance of 2.5, 6 and 10 represent samples derived from the same strain, subspecies and species, respectively.

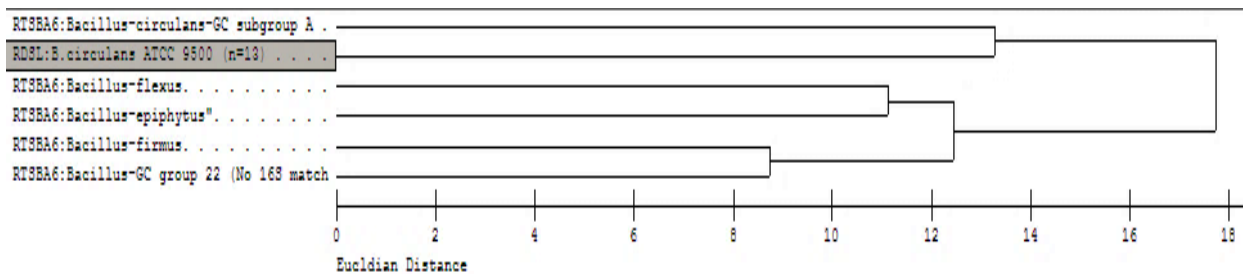
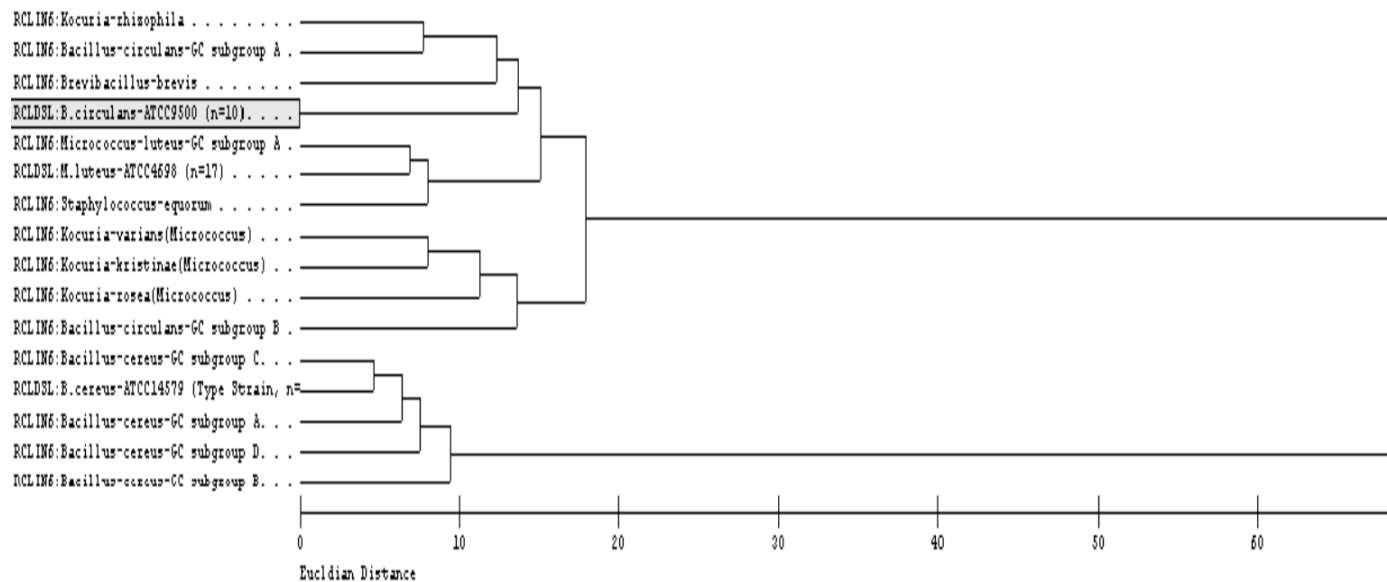
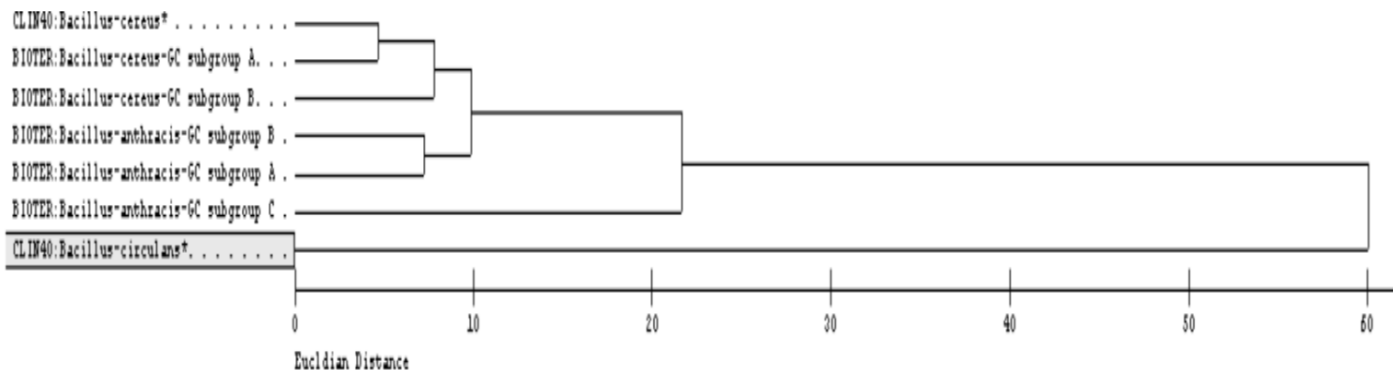


Figure B-1: Environmental database relationships for *Bacillus circulans* ATCC 9500



**Figure B-2: Clinical database relationships for Bacillus circulans ATCC 9500**



**Figure B-3: Bioterrorism database relationships for Bacillus circulans**

## Appendix C: The DSL *Bacillus circulans* strain 16S r RNA Gene Sequence Analysis

Table C-1: Matches to *B. circulans* strain ATCC 9500 in the MicroSeq® Full Gene Library v2.0 Sequence Match<sup>1</sup>

Match %	Sequence Entry
99.38	<i>Bacillus circulans</i> strain ATCC=4513
96.61	<i>Bacillus firmus</i> strain ATCC=14575
96.47	<i>Bacillus niacini</i> strain DSM=2923
96.23	<i>Bacillus flexus</i> strain ATCC=49095
95.89	<i>Bacillus megaterium</i> strain ATCC=14581
95.86	<i>Bacillus horikoshii</i> strain DSMZ=8719
95.82	<i>Bacillus cohnii</i> strain ATCC=51227
95.49	<i>Bacillus lentus</i> strain ATCC=10840

<sup>1</sup>The 16S ribosomal RNA gene sequence data has been generated by Health Canada's Healthy Environments and Consumer Safety Branch.

The 16S rRNA gene sequence of *B. circulans* the DSL strain was compared to the proprietary MicroSeq® Full Gene Library v2.0 Sequence Match.

## Appendix D: Antibiotic susceptibility of *Bacillus circulans*

**Table D-1: Antibiotic susceptibility of *B. circulans* based on human case reports**

Antibiotic	Resistant	Susceptible	Reference
Ampicillin	No	Yes	Alebouyeh et al. 2011; Banerjee et al. 1988; Gurol et al. 2007
Ampicillin	Yes	No	Fontana et al. 1997
Azlocillin	No	Yes	Banerjee et al. 1988
Amikacin	No	Yes	Berry et al. 2004; Castagnola et al. 1997; Fontana et al. 1997
Amoxicillin	No	Yes	Goudswaard et al. 1995; Krause et al. 1999
Amoxicillin /clavulanate	No	Yes	Fontana et al. 1997
Aztreonam	No	Yes	Alebouyeh et al. 2011
Cephadrine 1	Yes	No	Goudswaard et al. 1995
Cefazolin 1	Yes	No	Alebouyeh et al. 2011; Weber et al. 1988 <sup>a</sup>
Cefazolin 1	No	Yes	Fontana et al. 1997
Ceftazidime 3	Yes	No	Castagnola et al. 1997; Weber et al. 1988 <sup>a</sup>
Ceftiofur 3	Yes	No	Alebouyeh et al. 2011
Cefotaxime 3	Yes	No	Berry et al. 2004; Goudswaard et al. 1995
Cefuroxime 2	Yes	No	Goudswaard et al. 1995; Fontana et al. 1997
Chloramphenicol	Yes	No	Berry et al. 2004
Ciprofloxacin	No	Yes	Berry et al. 2004; Fontana et al. 1997; Goudswaard et al. 1995; Krause et al. 1999; Gurol et al. 2007; Weber et al. 1988 <sup>a</sup>
Clindamycin	Yes	No	Krause et al. 1999; Fontana et al. 1997
Clindamycin	No	Yes	Gurol et al. 2007
Cotrimoxazole	No	Yes	Fontana et al. 1997
Daptomycin (LY146032)	Yes	No	Weber et al. 1988 <sup>a</sup>
Erythromycin	No	Yes	Berry et al. 2004; Goudswaard et al. 1995;

<b>Antibiotic</b>	<b>Resistant</b>	<b>Susceptible</b>	<b>Reference</b>
			Fontana et al. 1997; Gurol et al. 2007
Fusidic acid	Yes	No	Krause et al. 1999
Gentamicin	No	Yes	Alebouyeh et al. 2011; Banerjee et al. 1988, Berry et al. 2004; Fontana et al. 1997; Krause et al. 1999
Levofloxacin	No	Yes	Gurol et al. 2007
Linezolid	No	Yes	Gurol et al. 2007
Imipenem	No	Yes	Banerjee et al. 1988; Berry et al. 2004; Castagnola et al. 1997; Weber et al. 1988 <sup>a</sup>
Kanamycin	No	Yes	Alebouyeh et al. 2011
Meropenem	Yes	No	Alebouyeh et al. 2011
Meropenem-EDTA	No	Yes	Alebouyeh et al. 2011
Methicillin	Yes	No	Alebouyeh et al. 2011
Metronidazole	Yes	No	Goudswaard et al. 1995
Netilmicin	No	Yes	Castagnola et al. 1997; Fontana et al. 1997
Oxacillin	Yes	No	Castagnola et al. 1997; Fontana et al. 1997; Weber et al. 1988 <sup>a</sup>
Penicillin	Yes	No	Alebouyeh et al. 2011; Banerjee et al. 1988; Berry et al. 2004; Weber et al. 1988 <sup>a</sup>
Penicillin	No	Yes	Goudswaard et al. 1995; Gurol et al. 2007
Piperacillin	No	Yes	Banerjee et al. 1988; Castagnola et al. 1997
Piperacillin	Yes	No	Alebouyeh et al. 2011
Piperacillin-tazobactam	Yes	No	Alebouyeh et al. 2011
Piperacillin-tazobactam	No	Yes	Berry et al. 2004
Rifampicin	No	Yes	Krause et al. 1999; Fontana et al. 1997
Rifampin	No	Yes	Gurol et al. 2007
Sulphametaxazole	No	Yes	Gurol et al. 2007
Teicoplanin	Yes	No	Fontana et al. 1997
Teicoplanin	No	Yes	Gurol et al. 2007
Telcoplanin	No	Yes	Castagnola et al. 1997; Krause et al. 1999

<b>Antibiotic</b>	<b>Resistant</b>	<b>Susceptible</b>	<b>Reference</b>
Telitromycin	No	Yes	Gurol et al. 2007
Tetracycline	No	Yes	Alebouyeh et al. 2011
Ticarcillin	No	Yes	Banerjee et al. 1988; Castagnola et al. 1997
Tobramycin	No	Yes	Castagnola et al. 1997
Trimethoprim	No	Yes	Krause et al. 1999; Gurol et al. 2007
Vancomycin	No	Yes	Banerjee et al. 1988; Berry et al. 2004; Castagnola et al. 1997; Goudswaard et al. 1995; Krause et al. 1999; Gurol et al. 2007; Weber et al. 1988 <sup>a</sup>
Vancomycin	Yes	No	Alebouyeh et al. 2011, Fontana et al. 1997

<sup>a</sup> Available information pertains to the type strain.