Government of Canada Gouvernement du Canada

Final Screening Assessment of *Pseudomonas* sp. ATCC 13867

Environment and Climate Change Canada

Health Canada

May 2016

Canada

Cat. No.: En14-250/2016E-PDF
ISBN 978-0-660-05037-9

Synopsis

Pursuant to paragraph 74*(b)* of the *Canadian Environmental Protection Act, 1999* (CEPA 1999), the Minister of the Environment and Climate Change and the Minister of Health have conducted a screening assessment on *Pseudomonas sp.* ATCC[1] 13867.

Pseudomonas sp. ATCC 13867 belongs to a group of strains that are currently without a validated species name. Prior to 1982, the species was referred to as '*Pseudomonas denitrificans'* before that name was officially rejected. For the purposes of this assessment, the name "*Pseudomonas sp.* ATCC 13867" will be used when information pertains specifically to this strain.

Pseudomonas sp. ATCC 13867 is a bacterium that can proliferate in soil and water. It has properties that make it of potential use in the production of vitamin B_{12}, coenzyme Q, other biochemicals and biofuels; in denitrification products for use in soil improvement, in treatment of activated sludge and wastewater and oil degradation.

No adverse effects in terrestrial or aquatic plants, invertebrates or vertebrates or infections in humans have been attributed to *Pseudomonas sp.* ATCC 13867 or its close relatives.

This assessment considers the aforementioned characteristics of *Pseudomonas sp.* ATCC 13867 with respect to environmental and human health effects associated with consumer and commercial product use and industrial processes subject to CEPA 1999, 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 1999, 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 *Pseudomonas sp.* ATCC 13867 was not imported into or manufactured in Canada in 2008.

Considering all available lines of evidence presented in this screening assessment, there is low risk of harm to organisms and the broader integrity of the environment from *Pseudomonas* sp. ATCC 13867. It is concluded that *Pseudomonas* sp. ATCC 13867 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.

Based on the information presented in this screening assessment, it is concluded that *Pseudomonas* sp. ATCC 13867 does not meet the criteria under paragraph

[1] America Type Culture Collection

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 1999), the Minister of the Environment and Climate Change 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 1999)[2]. *Pseudomonas* sp. ATCC 13827 was added to the DSL under Section 105(1) of CEPA 1999 because it was manufactured in or imported into Canada between January 1, 1984, and December 31, 1986 and it entered or was released into the environment without being subject to conditions under CEPA 1999 or any other federal or provincial legislation.

This Screening Assessment considers hazard information obtained from the public domain and from unpublished research data, as well as comments from scientific peer reviewers. Exposure information was obtained from the public domain and from a mandatory CEPA 1999 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 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 the DSL-listed strain *Pseudomonas* sp. ATCC 13867 are identified as such. Strain-specific data was obtained from several sources: the Nominator, the American Type Culture Collection (ATCC), and unpublished data generated by Health Canada[3] and the scientific literature. Where strain-specific data were not available, surrogate information from the literature was used. When applicable, literature searches conducted on the organism included its synonyms and superseded names. Literature searches were conducted using scientific literature databases (SCOPUS, CAB Abstracts, and NCBI PubMed), web searches, and key search terms for the identification of human health and environmental hazards. Information identified up to August 2014 was included in this Screening Assessment Report.

[2] A determination of whether one or more criteria of section 64 of CEPA 1999 are met is based upon 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 1999 on *Pseudomonas* sp. ATCC 13867 is not relevant to, nor does it preclude, an assessment against the hazard criteria for Workplace Hazardous Materials Information System (WHMIS) that are specified in the *Controlled Products Regulations* for products intended for workplace use.

[3] Testing conducted by Health Canada's Environmental Health Science and Research Bureau

Decisions from Domestic and International Jurisdictions

The Public Health Agency of Canada (PHAC) assigned the species known as *Pseudomonas* sp. to Risk Group 1 (low individual and community risk) for both humans and terrestrial animals (personal communication, PHAC 2014).

The Canadian Food Inspection Agency (CFIA) does not consider the species known as *Pseudomonas* sp. to be an animal pathogen (personal communication, CFIA 2014). Some members of the *Pseudomonas* genus are considered to be important plant pathogens; however, in the case of *Pseudomonas* sp. ATCC 13867, it was not possible to determine whether or not it is a pathogenic strain. Therefore, if the organism was to be imported, plant pest containment 1 (PPC-1) would be required for work with this organism (personal communication, CFIA 2014).

No other regulatory decisions by other international governments or organizations[4] were identified for *Pseudomonas* sp. ATCC 13867 despite the numerous current and potential uses identified.

[4] Government agencies and organizations searched include the US Environmental Protection Agency; US Food and Drug Administration; World Health Organization; US Center for Disease Control; Australian Department of Health; European Food Safety Authority; European Agency for Safety and Health at Work; European Food Standards Agency; Joint FAO/WHO Expert Committee on Food Additives.

1. Hazard Assessment

1.1 Characterization of *Pseudomonas* sp. ATCC 13867

1.1.1 Taxonomic identification and strain history

Binomial name: *Pseudomonas* sp.

Taxonomic designation:

Kingdom:	Bacteria
Phylum:	Proteobacteria
Class:	Gammaproteobacteria
Order:	Pseudomonadales
Family:	*Pseudomonadaceae*
Genus:	*Pseudomonas*
Species:	*Pseudomonas* sp. (Doudoroff et al. 1974; JCICSB, 1982)
Strain:	ATCC 13867

Other strain designations of *Pseudomonas* sp. ATCC 13867 include 926, NCIB 9496, NCIMB 9496 BCRC 14386, CCRC 14386, CCT 5425, CCUG 1783, CIP 104375, DSM 1650, DSM 1650/5040, Hugh 926, IAM 12573, IFO 13302, JCM 20650, LMD 84.60, LMG 7983, NBIMCC 1625, NBRC 13302 (Delwiche, 1959; Doudoroff et al. 1974; Sacks and Barker, 1952).

1.1.1.1 Synonyms and superseded names

Pseudomonas denitrificans (Lysenko, 1961; Peix et al. 2009), *Pseudomonas nitroreductans* Iizuka and Komagata 1964 emend. Lang et al. 2007 (DSMZ, 2014); *Pseudomonas multiresinivorans* (Mohn et al. 1999; DSMZ, 2014), *Bacillus denitrificans fluorescens* Christensen, 1903 (Lysenko, 1961).

Strain ATCC 13867 has historically been referred to as '*P. denitrificans*'; however, the species name was officially rejected in 1982 as *nomen ambiguum* based on a phenotypic and limited genotypic characterization (Doudoroff et al. 1974; JCICSB, 1982). In spite of the rejected status of the species name, the DSL strain (ATCC 13867) and another strain (ATCC 19244) are often still referred to as '*P. denitrificans*' in the literature. However, these two strains represent different species that can be differentiated by DNA composition and at least 40 phenotypic characteristics (Doudoroff et al. 1974). Therefore, information specific to the DSL strain will be identified with the name "*Pseudomonas* sp. ATCC 13867" (regardless of the name used by the source). Other strains historically named '*P. denitrificans*' may be distantly related to the DSL strain, and are therefore not considered reliable as surrogates; however, to be protective of the environment and human health, reports of adverse effects attributed to '*P. denitrificans*' were considered in this assessment where there is a possibility that the implicated strain could be a close relative of the DSL strain.

1.1.1.2 Strain history of *Pseudomonas* sp. ATCC 13867

Pseudomonas sp. ATCC 13867 was isolated from soil after enrichment with succinate-nitrate medium (Sacks and Barker, 1949). Originally named *Pseudomonas denitrificans* Bergey et al. strain 926 or Hugh 926, it was deposited in to the American Type Culture Collection (ATCC) by R. Hugh who had received the strain from C. Delwiche (Table 1-1).

Table 1-1: Major culture collections where *Pseudomonas* sp. ATCC 13867 was deposited

Culture collection	Strain designation	Year deposited
American Type Culture Collection	ATCC 13867	Not available
National Collection of Industrial, Food and Marine Bacteria	NCIMB 9496	1963
Deutsche Sammlung von Mikrooganismen und Zellkulturen	DSM 1650 (historical number: DSM 50405)	Not available
Institute for Fermentation	IFO 13302 (=NBRC[a] 13302)	Not available
IAM Culture Collection	IAM 12573	Not available
Japan Collection of Microorganisms	JCM 20650	2007

[a] NITE Biological Resource Center

1.1.1.3 Phylogeny of the genus *Pseudomonas*

Members of the *Pseudomonas* genus are Gram negative, diverse, widely distributed and dominated by non-pathogenic, saprophytic colonizers of soil, water, and rhizosphere ecosystems and non-pathogenic commensal colonizers of healthy human skin (Cogen et al. 2008; Li et al. 2013a). Historically, the genus *Pseudomonas* (*sensu lato*) included members from the alpha, beta, gamma-beta and gamma proteobacteria, many of which have been or are likely to be reclassified based on modern taxonomic methods. A subgroup of *Pseudomona*s species within the gamma proteobacteria are considered to represent the genus (*sensu stricto*). These include the *P. aeruginosa*, *P. chloroaphis*, *P. fluorescens*, *P. putida*, *P. stutzeri* and *P. syringae* species groups.

A phylogenetic tree was constructed using publicly available 16S rRNA gene sequences and sequences obtained from Anzai et al. (2000) (Appendix 1, **Error! Reference source not found.**). The DSL strain (named *Pseudomonas denitrificans* ATCC 13867 in Figure 1-1) is distinct from '*P. denitrificans'* neotype strain ATCC 19244 (named *Pseudomonas denitrificans* IAM 12023 in Figure 1-2) as a separate species and clusters differently in phylogenetic analysis (Appendix 1, **Error! Reference source not found.**). *Pseudomonas* sp. ATCC 13867 does not fit within the *P. aeruginosa* group (Figure 1-1), whereas '*P. denitrificans'* ATCC 19244 does (Figure 1-2).

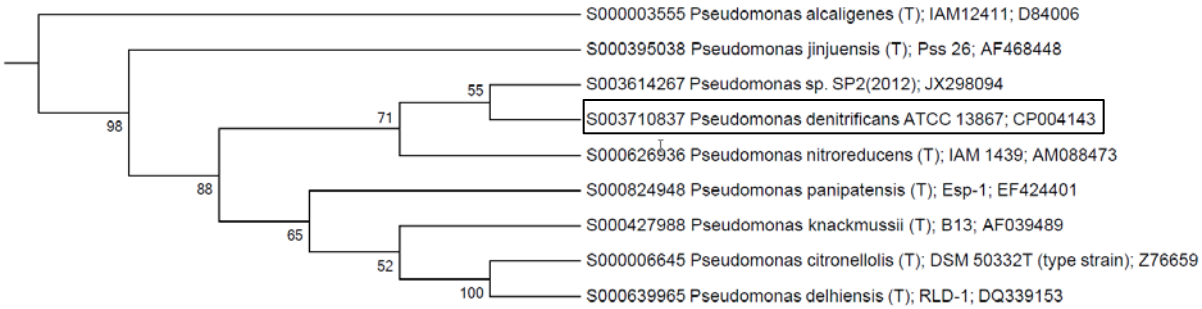

Figure 1-1: Phylogenetic subtree tree using 16S rRNA gene sequences showing species and strains that are closely related to *Pseudomonas* sp. ATCC 13867

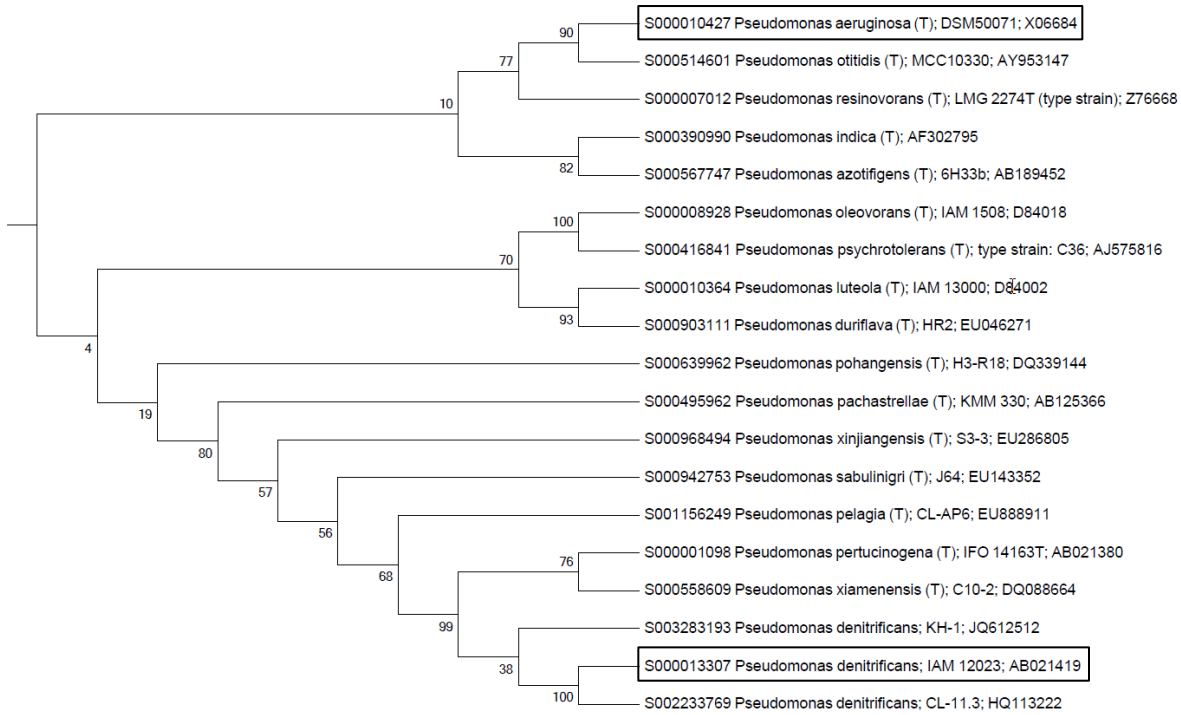

Figure 1-2: Phylogenetic subtree tree using 16S rRNA gene sequences showing species and strains that group closely with the pathogenic species *P. aeruginosa*

1.1.1.4 Phenotypic and molecular characteristics

The purpose of this section is to describe characteristics that can be used to distinguish between *Pseudomonas* sp. ATCC 13867, a closely related strain (SP2, see Figure 1-1) and *Pseudomonas aeruginosa* PAO1, as a representative pathogenic relative (Observations by Health Canada scientists for ATCC 13867 were consistent with those reported in the literature for most morphological and growth characteristics. Although growth curves were not generated at Health Canada, it was observed that colony size of *Pseudomonas* sp. ATCC 13867 varied depending on the temperature in which it was incubated when plated on tryptic soy

broth for 48 hours (1-2 mm at 28°C, 1 mm at 32°C, 3 mm at 37°C and 0.5 mm at 42 °C). This finding could suggest an optimal growth rate closer to 37°C.

Table 1-2 to Table 1-4). Observations by Health Canada scientists for ATCC 13867 were consistent with those reported in the literature for most morphological and growth characteristics. Although growth curves were not generated at Health Canada, it was observed that colony size of *Pseudomonas* sp. ATCC 13867 varied depending on the temperature in which it was incubated when plated on tryptic soy broth for 48 hours (1-2 mm at 28°C, 1 mm at 32°C, 3 mm at 37°C and 0.5 mm at 42 °C). This finding could suggest an optimal growth rate closer to 37°C.

Table 1-2: Morphological and growth characteristics of *Pseudomonas* sp. ATCC 13867, *Pseudomonas* sp. SP2 and *P. aeruginosa* PAO1

Characteristic[a]	ATCC 13867	Strain SP2	Strain PAO1
Cell size (L × W)	1.05 × 0.8 µm	2.0 × 0.5 µm	1.3 to 3.0 × 0.5 to 0.8 µm
Fluorescence	Non fluorescent	Non fluorescent	Fluorescent
Colonies	Entire, smooth, glistening, translucent, off-white/tan[b], raised[b]	Light cream colour	Entire, smooth, glistening, convex, and tan in colour with a fried egg morphology
Pigment production	None to pink	Pink	Diffusible, green
Optimal growth temperature	25°C	No data	37°C
Growth at 40°C	Negative	No data	Positive
Growth at 42°C	Negative[b]	No data	Positive

[a] Information compiled from Arasu et al. 2013; ATCC, 2013a; ATCC, 2013b; Doudoroff et al. 1974; Lysenko, 1961; Madigan et al. 2009; Palleroni, 1957; Palleroni, 2005; Pier et al. 2004; and Robertson et al. 1989.
[b] Unpublished data generated by Health Canada's Healthy Environments and Consumer Safety Branch

Table 1-3: Biochemical characteristics of *Pseudomonas* sp. ATCC 13867, *Pseudomonas* sp. SP2 and *P. aeruginosa* PAO1

Characteristic	ATCC 13867[a]	ATCC 13867[b]	Strain SP2[b]	Strain PAO1[b]
Gelatin liquefaction	Not observed	Negative	Negative	Positive
Utilization of maltose	Variable	Positive	Positive	Negative

a Unpublished data generated by Health Canada's Healthy Environments and Consumer Safety Branch
b (Arasu et al. 2013; Doudoroff et al. 1974; Lysenko, 1961; Palleroni, 2005)

The complete genome of *Pseudomonas* sp. ATCC 13867 has been sequenced; it is 5.7 Mbp in size and contains 5,135 genes (Winsor et al. 2011), 2,567 operons, and 5,059 protein-encoding genes (Ainala et al. 2013). No plasmids were identified in ATCC 13867 using the Pseudomonas Genome Database (Winsor et al. 2011).

Table 1-4: Molecular characteristics of *Pseudomonas* sp. ATCC 13867, *Pseudomonas* sp. SP2 and *P. aeruginosa* PAO1

Characteristic[a]	ATCC 13867	Strain SP2	Strain PAO1
G+C content	65.2%[a]	Not available	66.6%[a]
Genome size and accession number	5.7 Mb (CP004143)	Not available	6.2 Mb (AE004091)
16S rRNA	NR_102805	JX298094	AB626118

[a] (Palleroni 2005; Winsor et al. 2011)

Characteristics such as cell morphology, total lipid content and sensitivity to certain antibiotics could permit distinction between *Pseudomonas* sp. ATCC 13867 and *Pseudomonas* sp. strain SP2 (Arasu et al. 2013). The differences highlighted in Table 1-5 can be used to distinguish between the DSL strain and other strains of *Pseudomonas* sp. Fatty acid methyl ester analysis of *Pseudomonas* sp. ATCC 13867 was conducted by Health Canada scientists (Appendix 2).

Table 1-5: Differentiation of *Pseudomonas* sp. ATCC 13867 and SP2 using cell morphology, fatty acid composition and antibiotic sensitivity adapted from Arasu et al. (2013)

Characteristic	*Pseudomonas* sp. ATCC 13867	*Pseudomonas* sp. SP2
Total lipid (%)	48%	50%
Cell morphology	Short cell shape with smoother surface	Elongated cell shape with rougher surface
Nalidixic acid	Sensitive	Intermediate
Sparfloxacin	Sensitive	Intermediate
Colistin	Intermediate	Resistant

1.1.2 Biological and ecological properties

1.1.2.1 Growth parameters

Pseudomonas sp. ATCC 13867 is a colourless sulphur bacterium with a diverse metabolism (Robertson et al. 1989). It is facultatively anaerobic, facultatively chemolithotrophic, and capable of both heterotrophic nitrification and aerobic denitrification (Kornaros and Lyberatos, 1998; Robertson et al. 1989). It is able to use a variety of carbon sources and will switch from a preferred one to a secondary source of carbon in a pattern of diauxic growth (Casasus et al. 2005; Hamilton et al. 2005). A maximum growth rate of 0.043 mg dry biomass/mL per hour was achieved for *Pseudomonas* sp. ATCC 13867 in 4 g/L glucose (Apel and Turick, 1993). The DSL strain is also able to use succinate, yeast extract, ethanol and pretreated sewage sludge as carbon and energy sources (Dasu et al. 1993; Nilsson et al. 1980; Wu et al. 2001). *Pseudomonas* sp. ATCC 13867 does not grow at 4^{o}C or 40^{o}C (Doudoroff et al. 1974). Growth kinetics and growth in different media of *Pseudomonas* sp. ATCC 13867 are presented in Appendices 3 and 4, respectively.

1.1.2.2 Persistence and survival in the environment

In one study, amplified fragment length polymorphisms (AFLP)-derived strain-specific DNA markers were developed to detect *Pseudomonas* sp. ATCC 13867 and other *Pseudomonas* species (Xiang et al. 2010). Using these markers in combination with quantitative real-time PCR, estimated concentrations of *Pseudomonas* sp. ATCC 13867 cells in soil could be tracked over time. Cell suspensions of *Pseudomonas* sp. ATCC 13867 were added to agricultural soil in the laboratory to a final concentration of 10^8 to 10^{10} CFU/g dry weight. *Pseudomonas* sp. ATCC 13867 persisted in clay loam soil (22^{o}C, pH 5.8, 60% water holding capacity) for at least 181 days, indicating a high capacity for colonization. The authors suggest that the long-term persistence observed for *Pseudomonas* sp. ATCC 13867 (longer than *P. aeruginosa*, and as long as *P. stutzeri*) may be attributed to the metabolic versatility of the species, and particularly its capacity for aerobic denitrification (Xiang et al. 2010).

In another study, *Pseudomonas* sp. ATCC 13867 formed biofilms in pressurized flow-through columns. The columns contained diatomaceous mudstone and sandstone, synthetic groundwater and were supplemented with sodium acetate. Cell numbers increased over the experimental period of 28 days (Harrison et al. 2011; Wragg et al. 2012).

On the basis of these studies, introduced populations of *Pseudomonas* sp. ATCC 13867 are expected to persist and proliferate under certain environmental conditions and where nutrients are available.

1.1.2.3 Transformation of nitrate and nitrite

The DSL strain was reported to grow more rapidly with both nitrate and oxygen together compared to either electron acceptor separately (Robertson et al. 1989). The DSL strain was able to rapidly reduce N_2O to N_2; maximum removal N_2O rate of 0.017 mM/hr/mg dry biomass occurred at 35˚C and an initial N_2O concentration of 0.9 mM (Apel and Turick, 1993). Above this concentration the reduction rate decreases (Apel and Turick, 1993). When *Pseudomonas* sp. ATCC 13867 was grown in nitrate or nitrite, N_2 gas was immediately produced (Robertson et al. 1989). The ability of *Pseudomonas* sp. ATCC 13867 to reduce nitrates and produce gas was also observed by Health Canada scientists.

1.1.2.4 Resistance to antibiotics, metals and other chemical agents

Pseudomonas sp. ATCC 13867 contains multiple genes for resistance to antibiotics, metals and other chemical agents (Winsor et al. 2011). These genes include:

- metallo-beta-lactamase family protein and beta-lactamase/D-alanine carboxypeptidase (*ampC*);
- efflux pumps, including the resistance-nodulation-cell division (RND)-type multidrug efflux system (genes including *MexR*, *RND*, *SugE*, identified), a fusaric acid resistance domain protein;

- aminoglycoside/hydroxyurea antibiotic resistance kinase;
- cobalt/zinc/cadmium resistance protein CzcA;
- glyoxalase/bleomycin resistance protein/dioxygenase;
- copper resistance protein A;
- organic hydroperoxide resistance protein; and
- phosphinothricin N-acetyltransferase (herbicide resistance) protein.

The antibiotic susceptibility profile of the DSL strain was recently reported (Arasu et al. 2013). When tested using the disk diffusion method, *Pseudomonas* sp. ATCC 13867 was sensitive to representatives of the major classes of antibiotics including aminoglycosides, β-lactamase inhibitors and fluoroquinolones; resistance was reported for carboxypenicillins and variable susceptibility was reported for cephalosporins (Table 1-6). The zone of inhibition was measured after incubation at 37^{o}C for 17 hours.

Table 1-6: Antibiotic susceptibility patterns of *Pseudomonas* sp. ATCC 13867 (using the disc diffusion method), adapted from Arasu et al. (2013)

Antibiotic (µg)	Diameter of inhibition zone (mm)	Status[a]
Amikacin (30)	23	Sensitive
Gentamicin (10)	25	Sensitive
Kanamycin (30)	34	Sensitive
Streptomycin (30)	17	Resistant
Tobramycin (10)	31	Sensitive
Penicillin (50)	0	Resistant
Ampicillin (50)	0	Resistant
Augmentin (30)	28	Not available[b]
Imipenem (10)	30	Sensitive
Ticarcillin (75)	25	Sensitive
Ciprofloxacin (5)	39	Sensitive
Gatifloxacin (5)	32	Sensitive
Levofloxacin (5)	37	Sensitive
Moxifloxacin (5)	30	Not available
Nalidixic acid (30)	35	Sensitive
Norfloxacin (10)	38	Sensitive
Ofloxacin (5)	34	Sensitive
Sparfloxacin (5)	32	Sensitive
Cefpodoxime (10)	0	Resistant
Ceftriaxone (30)	30	Sensitive
Colistin (10)	10	Intermediate
Co-Trimoxazole (25)	25	Not available

[a] According to the Clinical and Laboratory Standards institute (formerly NCCLS), USA.
[b] Interpretation was not available.

Antibiotic susceptibility testing was conducted by Health Canada scientists (Table 1-7). Most of the tested antibiotics effectively inhibited growth with the exception of trimethoprim.

Table 1-7: Antibiotic susceptibility of Pseudomonas sp. ATCC 13867

Antibiotic	*Pseudomonas* sp. ATCC 13867 (MIC, µg/mL)[a]	Result[b]
Amoxicillin	12	N/A[c]
Aztreonam	15±6	I[d] (S[e]≤1, R[f] > 16)
Cefotaxime/cephotaxime	7.5±3	N/A
Ciprofloxacin	0.37	S (S≤0.5, R>1)
Colistin	0.37	S (S≤4, R>4)
Doxycycline	1.5	N/A
Erythromycin	1.2	N/A
Gentamicin	0.37	S (S≤4, R>4)
Meropenem	0.37	S (S≤2, R>8)
Trimethoprim	24	N/A

[a] Unpublished data generated by Health Canada's Healthy Environments and Consumer Safety Branch.
[b] Based on EUCAST clinical breakpoints.
[c] N/A, not applicable. EUCAST indicated that susceptibility testing is not recommended as the species is a poor target for therapy with the drug and isolates may be reported as resistant without prior testing.
[d] I, intermediate susceptibility
[e] S, susceptible
[f] R, resistant

A strain obtained from C. Delwiche (original depositor of the DSL strain to ATCC), that is likely the DSL strain *Pseudomonas* sp. ATCC 13867, was used in a study that compared the inhibitory effect of heavy metals on pseudomonads. This strain was reported to be much more resistant to the effect of heavy metals compared to another isolate identified as *Pseudomonas* sp. and a strain of *P. aeruginosa* (Bollag and Barabasz, 1979).

No information was identified on the resistance of *Pseudomonas* sp. 13867 to disinfectants such as chlorine or quaternary ammonium compounds.

1.1.2.5 Pathogenic and toxigenic characteristics

Virulence factors of *P. aeruginosa* include pili; flagella; siderophores; pyocyanin; elastase; proteases; rhamnolipid; alginate; other polysaccharides; and lipopolysaccharides (reviewed in Hay et al. 2014; Nelson et al. 2002; Palleroni, 2005). The Pseudomonas Genome Database listing for *Pseudomonas* sp. ATCC 13867 confirms the presence of some of the same virulence factors (Winsor et al. 2011), including:

- genes involved with secretion including but not limited to type III secretion, type VI secretion protein IcmF, type IV secretion system protein and type I secretion membrane fusion protein (HlyD);
- genes associated with pilus assembly and modification, including but not limited to type VI secretion system pilus modification protein (PilV), pilus

assembly (PilZ), system protein, pilus biogenesis/stability protein (PilW) and pilus assembly protein (PilM);
- quorum sensing molecules: RNA polymerase-binding protein DksA, transcriptional regulator *MetR* and *TraR*/*DskA* family transcriptional regulator;
- siderophore proteins including iron siderophore sensor protein (FecR), TonB-dependent siderophore receptor, siderophore biosynthesis protein and Fe3+ siderophore ABC transporter periplasmic solute binding protein; and
- antibiotic biosynthesis monooxygenase, aminoglycosidase/hydroxyurea antibiotic resistance kinase, phosphinothricin N-acetyltransferase (antibiotic resistance) protein and antibiotic transporter.

A search of the literature and of the Pseudomonas Genome Database listing for *Pseudomonas* sp. ATCC 13867 confirms the absence of extracellular toxins, such as rhamnolipids, pyocyanin, pyochelin and hydrogen cyanide (Winsor et al. 2011)

The secreted exopolysaccharide alginate helps bacteria to adapt and survive in many habitats. It has also been implicated in biofilm and capsule formation and may increase resistance to antibiotics and bactericides and therefore enhance its ability to evade the host immune system (Govan and Deretic, 1996; reviewed in Hay et al. 2014; Rezaee et al. 2002). Several genes associated with alginate biosynthesis and regulation have been identified in *Pseudomonas* sp. ATCC 13867 including *alg8*, *alg44*, *algK*, *algE*, *algG*, *algX*, *algL*, *algJ*, *algF*, *algR* and *algB* (reviewed in Hay et al. 2014; Winsor et al. 2011). However, other genes required for alginate biosynthesis and regulation (such as *algD* (promoter), *algA*, *algU*, *algC* and *amrZ*) were not identified (reviewed in Hay et al. 2014; Winsor et al. 2011).

Biofilms have been extensively reported as a mechanism of pathogenicity in pseudomonads. Biofilms contribute to the persistence of infections and cells within them are up to 1,000 times more resistant to the effects of antimicrobial agents than their planktonic counterparts (O'Toole and Kolter, 1998; Costerton et al. 1999; Mah and O'Toole, 2001). *Pseudomonas* sp. ATCC 13867 is known to form biofilms (Harrison et al. 2011; Wragg et al. 2012).

Strong hemolytic activity (as well as lecithinase activity) may indicate the presence of cytotoxic phospholipases that may facilitate invasion and are associated with virulence (Rowan et al. 2001; Sorokulova et al. 2008). *Pseudomonas* sp. ATCC 13867 did not demonstrate any hemolysis when tested by Health Canada scientists.

Catalase activity can enable a micro-organism to protect itself from reactive oxygen-induced killing from immune cells, potentially making it a more effective pathogen. Catalase activity was determined by Health Canada scientists to be positive for *Pseudomonas* sp. ATCC 13867.

In testing conducted by Health Canada scientists, the cytotoxicity of *Pseudomonas* sp. ATCC 13867 was assessed in two cell lines, HT29 (human colonic epithelial cells) and J774A.1 (macrophage cells), with and without gentamicin. No toxicity was observed in HT29 cells. Some toxicity was observed in J774A.1 cells in the presence of gentamicin. In the absence of antibiotic, J774A.1 was also more

sensitive compared to HT29 (in general, J774A.1 cells are more sensitive to toxic substances). However, overall the toxicity is limited (maximum 30% bioreduction at 24 hours) suggesting that the structural components of the bacteria were able to cause a limited toxic response. Similar marginal responses were observed with some *Acinetobacter* species studied with the same MTT assay (Tayabali et al. 2012). In comparison, strains of the known pathogen, *P. aeruginosa*, caused a 60-90% drop in MTT formazan production.

1.1.3 Effects

To be precautionary and ensure that all cases of infection possibly involving *Pseudomonas* sp. ATCC 13867 or its close relatives would be identified, the following section also includes information on cases of infection attributed to *'P. denitrificans'*.

1.1.3.1 Environment

One study reported *'P. denitrificans'* as being widespread in populations of the plant nematode, *Xiphinema americanum*, in West Virginia. However, it was unclear whether the relationship was symbiotic or pathogenic in nature (Adams and Eichenmuller, 1963).

No data were identified specifically implicating *Pseudomonas* sp. ATCC 13867 or its close relatives *Pseudomonas* sp. SP2, *P. nitroreducens* or *P. citronellolis* in adverse effects in aquatic or terrestrial invertebrates, vertebrates, or plants.

1.1.3.2 Humans

Similarly, no information was identified implicating *Pseudomonas* sp. ATCC 13867 or its close relatives *Pseudomonas* sp. SP2, *P. nitroreducens* or *P. citronellolis* in adverse human health effects.

One fatal case implicated a strain of '*P. denitrificans'* as the etiological agent in a serious infection in an individual predisposed by underlying disease (Fischer et al. 1981). Pure cultures of *'P. denitrificans'* were isolated from cerebrospinal fluid in a case of fatal meningitis in a patient with systemic lupus erythematosus, chronic leg ulcers, mitral insufficiency, seizure disorder, and mild dementia. The authors proposed that an old healing ulcer led to bacteremia which ultimately resulted in the meninges becoming infected as well. Treatment in this case was compromised by resistance to a number of antibiotics. It is unclear whether the isolated micro-organism was closely or distantly related to the DSL strain.

A therapeutic bronchoscopy yielded '*P. denitrificans*' and *S. aureus* in a cystic fibrosis patient with a two-year history of bilateral recurrent lung abscesses (Canny et al. 1986). Despite treatment with tobramycin, ticarcillin, and cloxacillin, the patient developed septic shock with disseminated intravascular coagulation and compromised renal function resulting in death. '*P. denitrificans*' was co-isolated with *S. aureus* from the lung abscess of a patient with cystic fibrosis at autopsy, but blood

cultures yielded only '*P. denitrificans*' with an antibiotic susceptibility identical to the organism cultured from the lung abscess.

No cases of allergic reactions related to *Pseudomonas* sp. ATCC 13867 or its close relatives *Pseudomonas* sp. SP2, *P. nitroreducens* or *P. citronellolis* were found in the literature. Like all micro-organisms, the DSL strain contains or produces components, such as lipopolysaccharides and enzymes, which may act as immune stimulants, allergens or sensitizers. Sensitization or allergic reactions to micro-organisms could occur via dermal and respiratory routes in frequently exposed or susceptible individuals (Martel et al. 2010; Ring et al. 1992).

1.2 Hazard Severity

The environmental and human health hazard severity for *Pseudomonas* sp. ATCC 13867 is assessed to be low because 1) *Pseudomonas* sp. ATCC 13867 can be discriminated from closely related *Pseudomonas* species found in the environment and from *P. aeruginosa* strains that are pathogenic towards human and environmental species; 2) although genes which may confer virulence were identified in the genome of *Pseudomonas* sp. ATCC 13867, there is no indication that the DSL strain acts as a pathogen. It is possible that the identified virulence genes are inactive, or require the expression of other or missing genes to cause harm; 3) no adverse effects in environmental species or humans, attributed to the DSL strain or its close relatives, have been reported; and 4) in the unlikely event of infection, veterinary and clinical antibiotics are available.

Hazards related to micro-organisms used in the workplace should be classified accordingly under the Workplace Hazardous Materials Information System (WHMIS)[5].

2. Exposure Assessment

2.1 Sources of Exposure

This assessment considers exposure to *Pseudomonas* sp. ATCC 13867 resulting from its addition to consumer or commercial products or its use in industrial processes in Canada.

Pseudomonas sp. ATCC 13867 was nominated to the DSL in 2005 for its use in commercial and consumer products.

[5] A determination of whether one or more of the criteria of section 64 of CEPA 1999 are met is based upon 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 1999 on *Pseudomonas* sp. ATCC 13867 is not relevant to, nor does it preclude, an assessment against the hazard criteria for WHMIS that are specified in the *Controlled Products Regulations* for products intended for workplace use.

Responses to a voluntary questionnaire sent in 2007 to a subset of key biotechnology companies, combined with information obtained from other federal government regulatory and non-regulatory programs indicate that *Pseudomonas* sp. ATCC 13867 was in commercial use in 2006.

The Government conducted a mandatory information-gathering survey under section 71 of CEPA 1999, 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 *Pseudomonas* sp. ATCC 13867, whether alone, in a mixture, or in a product. No industrial, commercial or consumer activities using *Pseudomonas* sp. ATCC 13867 were reported in response to the section 71 Notice.

The 2007 and 2009 surveys differed significantly in target and scope. In this assessment, results from the 2009 survey were used to estimate exposure from current uses because it requested information on uses of the micro-organism strain that is listed on the DSL, whereas the 2007 survey asked about uses of the products that had been associated with the micro-organism at the time it was nominated to the DSL. Because product formulations may have changed, information from the 2009 survey may more accurately represent current uses. Uses reported in the 2007 voluntary survey were also considered in the assessment of potential uses.

Although no uses were reported for *Pseudomonas* sp. ATCC 13867 during the mandatory survey, it is available for purchase from the ATCC. As it is on the DSL, and so can be used in Canada without prior notification, it could be an attractive choice for commercialization. A search of the public domain (MSDS, literature and patents) revealed the following consumer, commercial and industrial applications of other strains *of Pseudomonas* sp. To ensure that all potential uses of the DSL strain were captured, activities identified for '*P. denitrificans*' were also included:

- production of vitamin B_{12} (Kang et al. 2012; Li et al. 2008a; Li et al. 2008b; Li et al. 2008c; Li et al. 2012; Li et al. 2013b), coenzyme Q (Aida et al. 1981), cobalamins (Blanche et al. 1991; Blanche et al. 1997) and commodity chemicals (Yoshikuni et al. 2010);
- biofuel production (Yoshikuni and Kashiyama, 2009);
- soil improvement through mineral precipitation by microbial denitrification (Ellis et al. 1996; Hamdan et al. 2011);
- treatment of activated sludge in municipal wastewater treatment plants (Copp and Dold, 1998);
- wastewater treatment (Shiotani et al. 1998);
- denitrification of water (Nilsson et al. 1980);
- oil degradation (Yumoto et al. 2005) with potential use for bioremediation of contaminated soils; and
- as part of a mixture of micro-organisms to reduce environmental/atmospheric pollution by reducing the concentration of nitric oxides, ammonia, fine dust and CO_2 from sources of combustion and decontamination of domestic and commercial settings (Valenti, 2006).

2.2 Exposure Characterization

2.2.1 Environment

Based on the absence of industrial, consumer and commercial activity in Canada according to the section 71 Notice, the overall environmental exposure estimation for *Pseudomonas* sp. ATCC 13867 is low. Nevertheless, given the range and scale of known and potential applications listed in Section 2.1, there is potential for an increase in environmental exposure to products containing *Pseudomonas* sp. ATCC 13867, and therefore potential exposure scenarios arising from these products have been considered.

Should potential uses identified in Section 2.1 be realized in Canada, plant and animal exposure to *Pseudomonas* sp. ATCC 13867 will depend on its persistence and survival in the environment. However, the DSL strain is metabolically versatile and is expected to readily colonize new terrestrial environments. Xiang et al. (2010) investigated the persistence of *Pseudomonas* sp. ATCC 13867 in microcosm soil and reported that populations of this strain persisted in soil for at least 181 days, indicating a high capacity for colonization. The authors suggest that the long-term persistence observed for *Pseudomonas* sp. ATCC 13867 may be attributed to the metabolic versatility of the species, and particularly its capacity for aerobic denitrification. In another study, mentioned in section 1.1.2.2, *Pseudomonas* sp. ATCC 13867 formed biofilms in pressurized flow-through columns in which the solid phase was diatomaceous mudstone and sandstone, and the liquid phase was synthetic groundwater supplemented with sodium acetate. Cell numbers increased over the experimental period of 28 days (Harrison et al. 2011; Wragg et al. 2012). Therefore, *Pseudomonas* sp. ATCC 13867 would be expected to be able to survive and persist in most terrestrial and aquatic environments.

The following exposure scenarios are based on known uses of other strains and probable future uses as described in Section 2.1. Uses such as bioremediation and water and wastewater treatment are likely to introduce *Pseudomonas* sp. ATCC 13867 to terrestrial ecosystems. Terrestrial invertebrates living in the soils at the site of application or disposal and plants growing in treated soils are likely to be the most directly exposed. Vertebrates could ingest *Pseudomonas* sp. ATCC 13867 while feeding on plants or invertebrates growing in treated or contaminated soils.

Aquatic and marine species may come into contact with *Pseudomonas* sp. ATCC 13867 from runoff subsequent to terrestrial application and from the direct application of *Pseudomonas* sp. ATCC 13867 to water bodies for uses such as water treatment (fresh and salt water), wastewater treatment, or disposal of wastewater from applications such as recovery of oil and metals or the manufacture of biochemicals and biofuels.

Aquatic applications could also expose terrestrial species. For example, grazing animals could ingest *Pseudomonas* sp. ATCC 13867 subsequent to its use in water

restoration, and plants and soil invertebrates could be exposed subsequent to the treatment of irrigation ponds.

In the event that consumer, commercial or industrial activities resume, the environmental exposure to *Pseudomonas* sp. ATCC 13867 will likely increase. The environmental compartments and species that will be exposed to the DSL strain will depend on the uses outlined in the exposure scenarios described above.

2.2.2 Human

Based on the absence of industrial, consumer or commercial activity in Canada according to the section 71 Notice, the overall human exposure estimation for *Pseudomonas* sp. ATCC 13867 is low. Nevertheless, given the range and scale of known and potential applications provided in Section 2.1, there is potential for an increase in human exposure to products containing *Pseudomonas* sp. ATCC 13867, and exposure scenarios arising from these products have been considered.

Should potential uses identified in Section 2.1 be realized in Canada, human exposure could be greatest through the use of consumer products intended for the treatment of aquariums and decorative ponds, degreasing of kitchen drains, cleaning and deodorizing of septic tanks, and composting. Handling and application of such products would be expected to result in direct exposure to the skin. Inhalation of aerosolized droplets or airborne dust containing *Pseudomonas* sp. ATCC 13867 generated during the application of such products could also occur.

Secondary to product application, residual *Pseudomonas* sp. ATCC 13867 on surfaces and in reservoirs such as treated drains could result in dermal exposure; oral exposure through inadvertent ingestion where the organism persists on food preparation surfaces; and exposure via inhalation where aerosols are generated (e.g., from kitchen garbage disposal units). Since *Pseudomonas* sp. ATCC 13867 is expected to persist following application, such exposures may be temporally distant from the time of application.

Should commercial products containing *Pseudomonas* sp. ATCC 13867 become available in Canada, the general population could be exposed as bystanders during commercial product application. 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, but in general is expected to be moderate.

Human exposure to bodies of water and soil treated with *Pseudomonas* sp. ATCC 13867, (e.g., through recreational activities), could also result in exposure of the skin and eyes, as well as inadvertent ingestion.

Indirect exposure to *Pseudomonas* sp. ATCC 13867 in the environment subsequent to its use in oil recovery, water and wastewater treatment, soil bioremediation, 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 no greater than direct exposure from the use of the organism in consumer products.

In the event that the organism enters municipal drinking water treatment systems through release from potential uses, the water treatment process, which utilizes one or more of the following methods: coagulation, flocculation, ozonation, filtration, ultraviolet radiation and chlorination, is expected to effectively eliminate these micro-organisms from drinking water.

In the event that the potential consumer, commercial or industrial uses of *Pseudomonas* sp. ATCC 13867 are realized, human exposure through the exposure scenarios described above can be expected and could include direct, possibly repeated, exposure to concentrated preparations of *Pseudomonas* sp. ATCC 13867.

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 *Pseudomonas* sp. ATCC 13867 to be low for both the environment and human health. Based on the absence of industrial, consumer or commercial activity in Canada according to the section 71 Notice, environmental and human exposure to *Pseudomonas* sp. ATCC 13867 is not currently expected (low exposure), so the risk associated with current uses is estimated to be low for both the environment and human health.

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

Pseudomonas sp. ATCC 13867 has properties that make it of interest for applications that could expose the environment and the general Canadian population to this strain in the future. The risk to the environment and human health from foreseeable future uses of *Pseudomonas* sp. ATCC 13867 is also low given that there is no evidence of adverse ecological effects or adverse effects to human health.

4. Conclusion

Based on the information presented in this screening assessment, it is concluded that *Pseudomonas sp.* ATCC 13867 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 *Pseudomonas sp.* ATCC 13867 does not meet any of the criteria set out in section 64 of CEPA 1999.

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Appendices

A. Phylogenetic tree

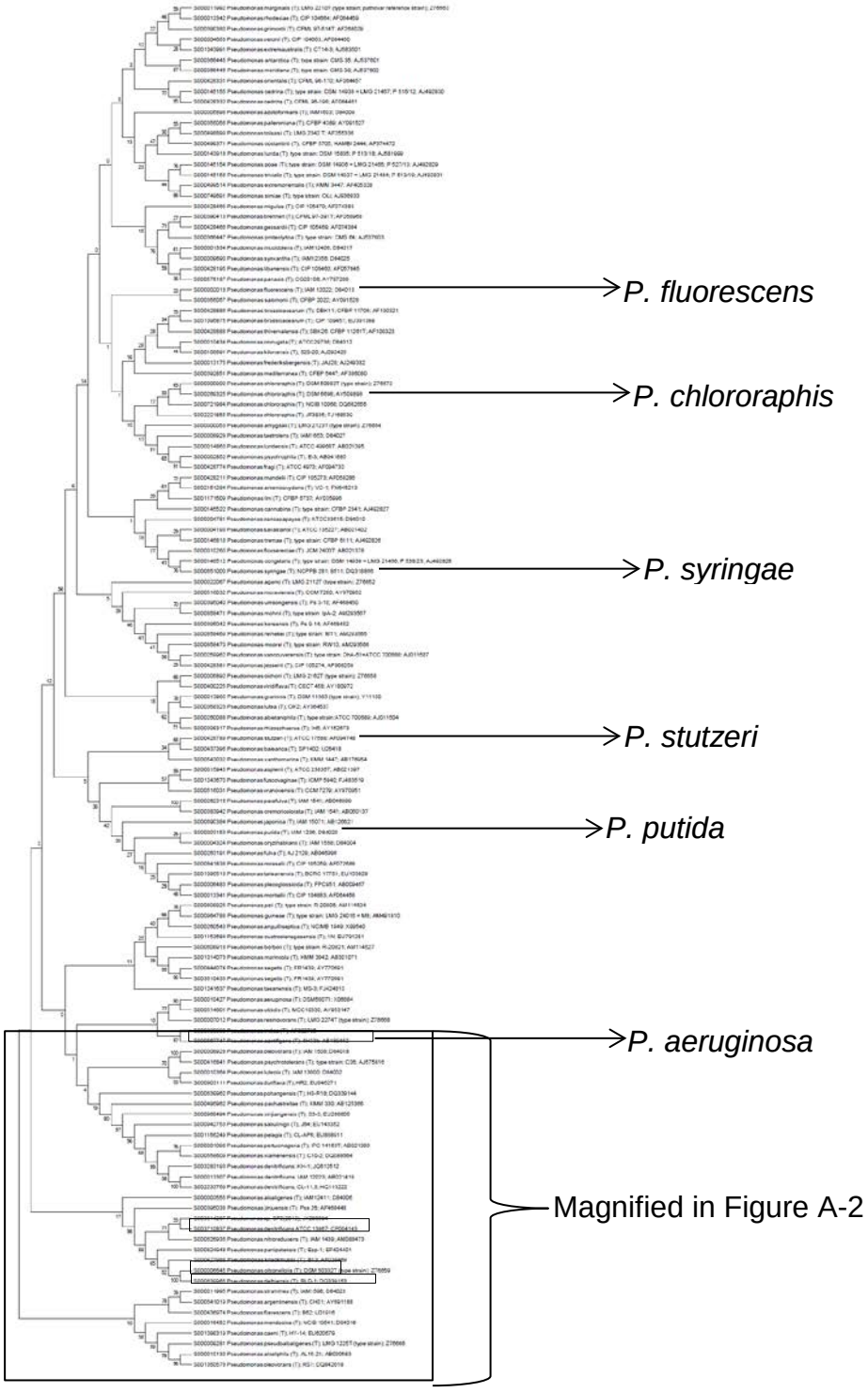

Figure 0-1: Phylogenetic tree using 16S rRNA gene sequences with species representing major groups within the *Pseudomonas* genus indicated

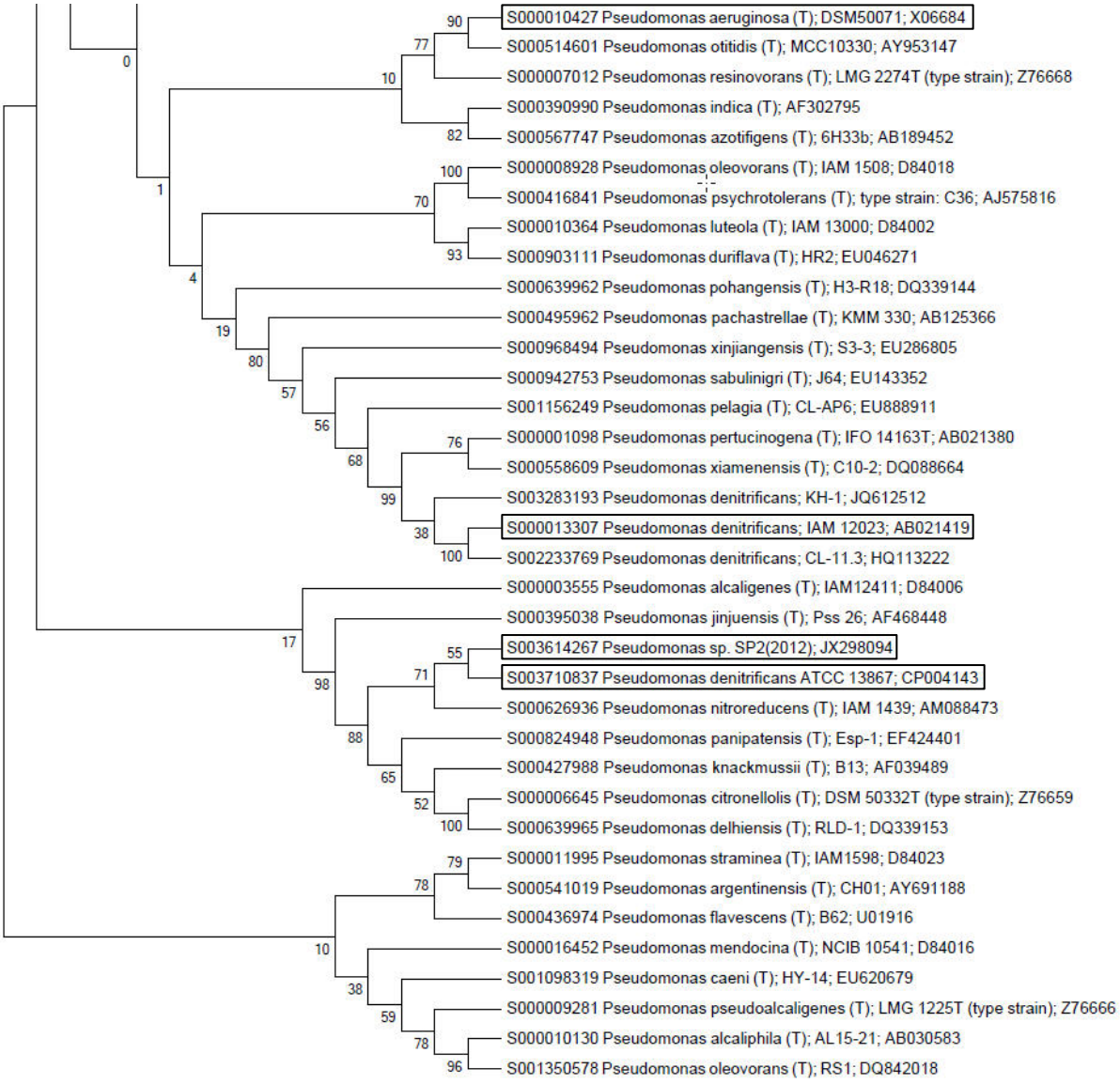

Figure 0-2: Partial phylogenetic tree using 16S rRNA gene sequences of *Pseudomonas* species

Phylogenetic trees were generated by the Environmental Health Science and Research Bureau using 16S rRNA gene sequences of the *Pseudomonas* genus (available via the Ribosomal Database Project http://rdp.cme.msu.edu/). The alignment was generated by Muscle and a Maximum Likelihood Tree was constructed with the Gamma distributed with invariant sites method and 500 bootstrap replicates, using the MEGA version 5.2 platform (Tamura et al. 2011). *Pseudomonas* sp. ATCC 18367, *Pseudomonas* sp. SP2, *P. denitrificans* ATCC 19244 and *P. aeruginosa* have been highlighted. In the phylogenetic tree the DSL strain appears as '*Pseudomonas denitrificans* ATCC 13867' and *Pseudomonas denitrificans* ATCC 19244 appears as '*Pseudomonas denitrificans* IAM 12023'.

B. Fatty Acid Methyl Ester (FAME) Analysis

Unpublished data generated by Health Canada's Healthy Environments and Consumer Safety Branch. Data presented based on clinical and environmental MIDI databases. MIDI is a commercial identification system that is based on the gas chromatographic analysis of cellular fatty acid methyl esters. The environmental and clinical databases showed the closest relationship of *Pseudomonas sp.* to *Pseudomonas aeruginosa*.

Table 0-1: Fatty Acid Methyl Ester (FAME) Analysis of *Pseudomonas* sp. ATCC 13867

Context	Environmental database	Clinical database
Frequency	6/6	5/5
Similarity index	0.485	0.847
First choice	Pseudomonas-aeruginosa-GC subgroup A	Pseudomonas-aeruginosa-mucoid strains

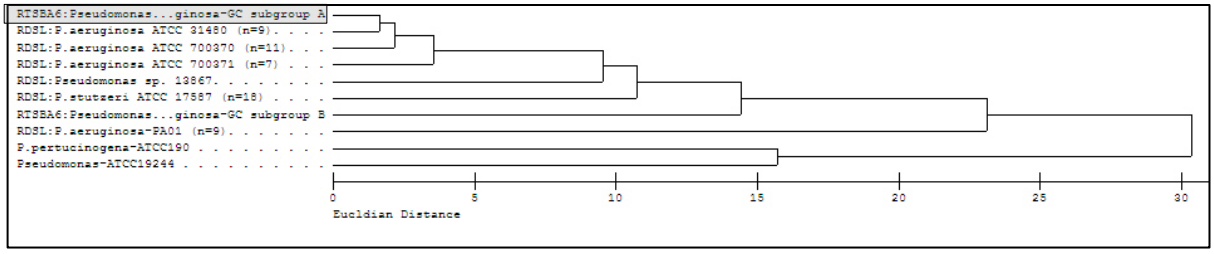

Figure 0-3: Environmental database nearest neighbor analysis and select strains

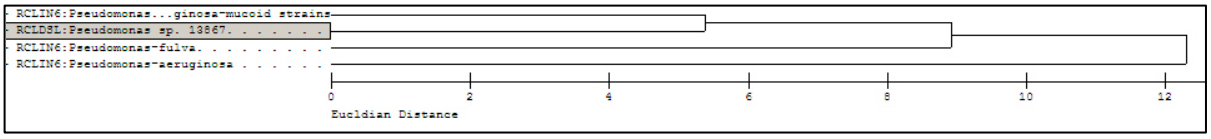

Figure 0-4: Clinical database nearest neighbor analysis

C. Growth Kinetics

Growth kinetics were investigated using Dulbecco's Modified Eagle Medium, Trypticase Soy Broth and Fetal Bovine Serum at various temperatures. Each table entry shows whether growth (increase in absorbance at 500nm) occurs at different temperatures (28, 32, 37, 42^{o}C).

Table 0-2: Growth Kinetics of *Pseudomonas* sp. ATCC 13867 in liquid media for 24 hours

Medium	28^{o}C	32^{o}C	37^{o}C	42^{o}C
Trypticase Soy Broth	+++[a]	+++	+[b]	-[c]
10% Fetal Bovine Serum (FBS)	~[d]	~	~	-
100% Fetal Bovine Serum	+	++[e]	-	-
Dulbecco's Modified Eagles Medium with FBS and glutamine	-	-	-	-

Unpublished data generated by Health Canada's Healthy Environments and Consumer Safety Branch. Optimal growth determined to be at 32^{o}C in liquid media.

[a] +++, strong growth (optical density (OD)>1)

[b] +, slow growth (OD>0.2)

[c] -, no growth (OD<0.05)

[d] ~, poor growth (OD<0.2)

[e] ++, modest growth (OD>0.5)

D. Growth in different media

Table 0-3: Growth of *Pseudomonas* sp. ATCC 13867 at 28°C (48 hours) in different media

Medium[a]	Results
Trypticase soy broth[a]	Positive
Cetrimide agar[b]	Positive
Starch[c]: Growth	Positive
Starch[c]: Hydrolysis	Weakly positive
Maconkey Agar[d]	Growth, non-pink colonies
Mannitol Egg Yolk Polymyxin supplements[e]	Negative
Mannitol Salt Agar[f]	Positive but alkaline reaction
Citrate utilization[g]	Negative[h]
Urea hydrolysis[i]	Negative

Unpublished data generated by Health Canada's Healthy Environments and Consumer Safety Branch
[a] All-purpose medium
[b] Selective for the growth of the Gram negative bacterium, *P. aeruginosa*
[c] Differential medium that tests the ability of an organism to produce extracellular enzymes that hydrolyze starch
[d] Detection of coliform organisms in milk and water; tests for ability of organism to ferment lactose
[e] *B. cereus* selective agar
[f] Isolation and differentiation of *Staphylococci*
[g] Ability to use citrate as the sole carbon source
[h] API results indicate that this should be positive for ATCC 13867
[i] Urea metabolism; screening of enteric pathogens from stool specimens