Risk Assessment Summary Conducted Pursuant to the New Substances Notification Regulations of the *Canadian Environmental Protection Act, 1999* Research Trials using *Pseudomonas putida* CR30RNSLL(pADPTel) NSN 10642

This document has been prepared to explain the regulatory decision taken under Part 6 of the *Canadian Environmental Protection Act, 1999* (CEPA 1999) regarding the experimental field study using *Pseudomonas putida* CR30RNSLL(pADPTel) by researchers from University of Saskatchewan, Carleton University, and the National Research Council.

P. putida CR30RNSLL(pADPTel) was notified pursuant to subsection 29.11(4) of the CEPA 1999 New Substances Notification (NSN) Regulations. The New Substances Branch of Environment Canada and the New Substances Assessment and Control Bureau of Health Canada have assessed the information submitted by the persons cited below and other scientific information available in order to determine whether *P. putida* CR30RNSLL(pADPTel) is *toxic** or capable of becoming toxic* as defined by section 64 of the CEPA 1999.

Regulatory Decision:

Based on the hazard and exposure considerations, the joint risk assessment conducted by Environment Canada and Health Canada concluded that *P. putida* CR30RNSLL(pADPTel) is not considered to be *toxic* to the Canadian environment or human health as described in section 64 of the CEPA 1999.

Introduction into the three experimental field study sites i) Carleton University in Ottawa, Ontario ii) Eugene Lods Agricultural Research Facility, MacDonald Campus McGill University in St. Anne de Bellevue, Quebec and (iii) Saskatchewan Wheat Pool Research Station in Saskatoon, Saskatchewan may proceed after July 8, 2001.

This evaluation does not include an assessment of human health risk in the occupational environment nor does it include an assessment of the potential exposure and risk to humans associated with the use of the organism in or as an item that falls under the purview of the *Food and Drugs Act*.

NSN Schedule:	XVII (Introduction in an experimental field study)
Organism Identity:	*Pseudomonas putida* CR30RNSLL(pADPTel*)*
Type of Organism:	Bacterium
Notifier(s):	J.J. Germida, University of Saskatchewan, C.W. Greer, National Research Council and R.C. Wyndham, Carleton University
Date of Decision:	July 8, 2001
Proposed use:	Laboratory production and introduction of *P. putida* CR30RNSLL(pADPTel) into three specific field trial sites to study the fate of the organism in soil and the plasmid that it contains for a three year period.

* In accordance with section 64 of the *Canadian Environmental Protection Act, 1999* (CEPA 1999) a substance is toxic if it is entering or may enter the environment in a quantity or concentration or under conditions that (a) have or may have an immediate or long-term effect on the environment or its biological diversity; (b) constitute or may constitute a danger to the environment on which life depends; or (c) constitute or may constitute a danger in Canada to human life or health.

Strain History / Genetic Modifications:

The parental strain *P. putida* CR30 is a naturally occurring micro-organism that was isolated from *Brassica napus* (canola) rhizosphere at the field study site at Carleton University in Ottawa. This strain was isolated on tryptone yeast extract (TYE) agar plates containing cyanuric acid (atrazine metabolite and nitrogen source). It was unambiguously identified based on BIOLOG, FAME, and 16S rRNA sequence analyses. CR30 has intrinsically high resistance to chloramphenicol and ampicillin, medium resistance to tetracycline and low resistance to rifampicin, naladixic acid and streptomycin.

The genetic modifications performed on *P. putida* CR30 allow the simultaneous tracking of the fate of the notified strain (using colorimetric lac and bioluminescent *lux* reporter genes) and its plasmid-encoded genes (*atz* herbicide degradation and *kilAtelAB* tellurite resistance), in soil. Resistance to tellurite, rifampicin and naladixic acid act as selectable markers for the organism. *P. putida* CR30RNSLL(pADPTel) was generated in the following manner:

1. *P. putida* CR30RNS, with high resistance to rifampicin (R), naladixic acid (N) and streptomycin (S), was derived from CR30 by selective pressure. This was achieved by growing the parent organism in TYE containing increasing concentrations of individual antibiotics to attain CR30R, CR30RN, and finally CR30RNS.
2. To transform CR30RNS to CR30RNSLL, the *lacZY-luxAB* gene cassette from plasmid pLLM1 (constructed using the method of Masson *et al.* 1993) was integrated into the CR30RNS chromosome. The *lacZY-luxAB* cassette is composed of the *Escherichia coli* beta-galactosidase (Z) and lactose permease gene (Y) (*lacZY*) (Barry 1988), the luciferase (*luxAB*) gene from *Vibrio harveyi* strain B392 (Miyamoto *et al.* 1985). The *lac-lux* cassette was integrated into the CR30RNS chromosome via Tn7 transposition. Southern blot analysis and restriction mapping showed the stable integration of the inserted genes into CR30RNSLL.
3. The pADP-1 plasmid was obtained from *Pseudomonas sp*. strain ADP, a soil isolate, collected at an atrazine herbicide distribution centre in Minnesota (Mandelbaum *et al.* 1995; De Souza *et al.* 1998). The plasmid contains the *atz* genes involved in the degradation of the herbicide atrazine to cyanuric acid. Other genes on pADP-1 are listed in Table 1. pADP-1 was modified to create pADPTel by the addition of the tellurite resistance operon (*kilAtelAB)* obtained from plasmid pDT1558 (Walter and Taylor 1989). The operon originated from *Klebsiella aerogenes* isolated from a patient in Birmingham, UK (Walter and Taylor 1992). Independent of step 2, a second aliquot of strain CR30RNS was transformed with the resulting pADPTel plasmid to yield CR30RNS(pADPTel). The presence of *kilAtelAB* in pADPTel was confirmed by Southern Blot.
4. The notified strain CR30RNSLL(pADPTel) was obtained by conjugal transfer of the pADPTel plasmid from CR30RNS(pADPTel) to CR30RNSLL. Transconjugates were grown on selective media containing glucose, atrazine (sole nitrogen source), tellurite, rifampicin, and naladixic acid and were identified by the production of a black pigment on the selective media and by bioluminescence in the presence of n-decanal.

Hazard Considerations:

Pathogenicity/Toxicity

In addition to the information provided by the notifiers, a review of in-house reference materials and a comprehensive search of the scientific literature were conducted to gather information on the potential harmful environmental and human health effects attributed to *P. putida* and the following strains that have been reclassified as *P. putida*: *P. ovalis, P. convexa, P. rugosa, P. striata, P. schuylkilliensis, P. eisenbergii, P. incognita* and *Arthrobacter siderocapsulatus.*

P. putida is very common in soils and plant rhizospheres and can also be isolated from water, plants, animal sources, hospital environments and human clinical specimens (OECD, 1997). The Office of Laboratory Security of the Public Health Agency of Canada designates *P. putida* as a Risk Group 1 organism. It is rarely infectious and is considered non pathogenic in humans. In a few isolated cases, *P. putida* has been identified as a source of secondary infections such septicemia, septic arthritis and bacteremia in susceptible and immunocompromised individuals (Anaissie *et al*., 1987; Romney *et al*., 2000; Yang *et al*., 1996). Despite their ubiquitous presence in the environment and their widespread use in research and environmental applications, reports of primary infections and allergic reactions attributed to *P. putida* in the scientific literature are rare.

P. putida has been reported to infect a wide variety of animals including goats, koalas, turkeys, geese, fish, turtles, cattle, horses, crayfish, snail, olive fly (OECD, 1997). In some of the reported cases, disease was attributed to other etiologic agents (Nishimori *et al*., 2000), or co-incidental with the presence of *P. putida* (Ladds *et al*., 1990; Cheng, 1986). It is believed to be a pathogen of potential concern to farmed and wild fish (OECD, 1997; Egusa, 1992), including yellowtails in Japan (Kusuda and Toyoshima, 1976). *P. putida* is not considered to be a finfish disease causing agent in Canada (Personal communication, Nathalie Bruneau, Department of Fisheries and Oceans, May 2001). The optimum growth temperature of the notified strain is 35°C, but it can grow at temperatures up to 41°C. Because most strains of *P. putida* are unable to grow at elevated temperatures, they are unlikely to be more than a rare opportunistic pathogen for warm-blooded animals (OECD, 1997). In an *in vivo* study conducted by George *et al*. (1999), the lungs and intestinal tract of male endotoxin-resistant mice administered intranasally with 1.17×10^7cfu *P. putida* were clear of the bacteria within two days post treatment. The same study also showed no mortality in the animals that received a single treatment of up to 1.2×10^9cfu *P. putida.*

P. putida is not considered to be a plant pathogen (Bradbury, 1986). A few strains, however, are known to produce pectate lyase, an enzyme involved in soft-rot disease of roots (Zolobowska and Pospieszny, 1999; Park *et al.,* 1997). The multiplication of *P. putida* in the rhizosphere of paddy rice plants has been implicated in “suffocation disease” (Bradbury, 1986). DNA probe tests conducted by the notifiers could not detect the presence of the pectate lyase gene in the notified strain.

Genetic Construction

Hazard considerations related to the inserted genetic materials were evaluated at each step in the cloning process. As shown in Table 1, some of the inserted genes cannot be replicated and/or expressed in the final construct. Those present in the final construct, including the antibiotic, tellurite, atrazine and the lac-lux selection markers, were found to pose little or no intrinsic hazard to the environment. None have the potential to be pathogenic, toxic, or toxicogenic to human health.

Table 1: Hazard considerations related to the genetic construction of the notified strain

Construct item	**Hazard**
PADPTel plasmid final construct:	
mer genes in pADPTel	Low. *mer* genes confer resistance to mercury ions which would otherwise be toxic to the organism. Organomercurials are no longer used therapeutically. Naturally present on pADP-1.

mobile elements (*tra* & *trb* operons) in pADPTel	No intrinsic hazard. Allows plasmid to transmit itself to a wide range of organisms via conjugation, thus transferring all genes on the plasmid. Naturally present on pADP-1..
oriV in pADPTel	No intrinsic hazard. Broad host range origin of replication, allows plasmid to be replicated in a wide variety of organisms. IncP plasmid viable in most Proteobacteria. Naturally present on pADP-1..
kilAtelAB operon in pADPTel (~3kb, verified to contain no extraneous DNA)	Low. Confers resistance to tellurite. Occurs in nature. Commercial use of tellurite is limited to use as part of an assay for the identification of coliform bacteria. Widespread tellurite resistance would render the tellurite component of current assays less useful.
atz genes in pADPTel	Low. Allows bacteria to degrade atrazine and grow when atrazine is the sole nitrogen source. Occurs in nature. Naturally present on pADP-1.
3 x IS1071 (tn3 IRs)	No intrinsic hazard. Short inverted repeat (IR) sequences recognized by *tnpA* and *tnpR* and allows transposition of DNA held between the IRs. Naturally present on APD-1.
Extra unknown genes on pADPTel from pADP-1 precursor	Unknown hazard. From a BLAST analysis, gene sequences were found to be homologous to transposases, ABC transporter genes, metabolic genes and other genes of unknown function. Could play a role in gene transfer.
CR30RNSLL final construct:	
Extraneous DNA in *luxAB* insertion cassette	Low. Verified with BLAST search of Tn7 insert sequence
Extraneous DNA in Tn7-*lacZY* insertion cassette	Low. Verified with BLAST search of Tn7 insert sequence
Resistance to 100μg/ml of Rifampicin, Nalidixic Acid and Streptomycin	Low. Confers resistance to antibiotics listed. Created by selective pressure. Located on chromosome. Likely due to point mutations (less-transferable) rather than plasmid-borne antibiotic resistance genes (more-transferable). Rifampicin used occasionally in animals, nalidixic acid is parent compound for fluoroquinolone class of antibiotics used therapeutically, streptomycin use limited. Resistance occurs in nature.
trp-lac-iucA promoter fusion	Low. It is a constitutive promoter. The sequence was analyzed with BLAST and no known hazards were found.
lacZY genes in chromosome	Low. Allows the breakdown of lactose and lactose analogs. Occurs in nature. Insertion into chromosome was confirmed by Southern Blot.
luxAB genes in chromosome	No intrinsic hazard. Renders bacteria bioluminescent. Insertion into chromosome was confirmed by Southern Blot.
Tn7 arms	No intrinsic hazard. Flanks *lacZYluxAB*. Non-motile unless Tn7 transposase genes are present.

Markers:
While ampicillin, tetracycline and chloramphenicol are considered to pose a moderate hazard according to the 1989 USEPA Biotechnology Science Advisory Committee, the field trial of the notified organism is not considered to compromise the therapeutic use of these antibiotics. Significant background levels of plasmid-mediated acquired resistance have been reported for all three of these antibiotics, which has limited their usefulness or effectiveness in veterinary medicine (Prescott, 2000). Given that there is no selective pressure for the transfer of these resistance genes, that the parent strain had the same high levels of resistance for all three antibiotics, and that the environmental exposure of the notified strain is expected to be limited to the three field trial sites, the human therapeutic and veterinary use of these antibiotics is not likely to be further compromised by the current experimental field study.

The antibiotic resistance genes for naladixic acid, streptomycin, rifampicin in *P. putida* CR30RNS are believed to have resulted from either point mutations in the genes that encode the enzymes that metabolize the antibiotics (Prescott, 2000; Walker, 2000; Glandorf *et al*., 1992), or due to changes in cell envelope permeability, and are therefore likely to be chromosomally encoded. This presents a greater level of stability than plasmid-born genes (Compeau *et al*., 1988). Resistance to these antibiotics is known to exist and be readily acquired (Safronova *et al.,* 1991; Adesiyun and Downes, 1999; Endtz *et al.,* 1991; Hosoda *et al.,* 1990). The design of the field trial will not provide a selective advantage to micro-organisms with resistance to these antibiotics. The use of these antibiotic resistance markers is not likely to compromise the human or veterinary therapeutic use of these antibiotics.

The use of heavy metal resistance markers is favoured over antibiotic resistance markers. The *kilAtelAB* operon from *K. aerogenes* confers resistance to tellurite. It provides colorimetric properties to the notified organism upon tellurite degradation on the culture media. The tellurite resistance gene is found in nature. Like the *atz* genes, the *kilAtelAB* gene is plasmid borne and is expected to be transferred to the surrounding bacteria in the field trial sites. Nevertheless, the presence of this gene is expected to pose low hazard.

Atrazine is a herbicide used extensively in North America. It is found in many aquatic (surface and groundwater) and terrestrial habitats influenced by agricultural pest management practices. Normal byproducts of atrazine degradation can be toxic, however, the notified strain was genetically modified to degrade atrazine to non-toxic byproducts. If degradation is not complete, some of the toxic by-products are expected to be metabolized by other soil bacteria (De Souza *et al.,* 1998) while others may accumulate in ways similar to other atrazine-contaminated soils described in the literature (Costa *et al.,* 2000; Topp *et al.,* 2000; Lerch *et al.,* 1999; Jones *et al.,* 1982).

Both the *lacZY* and the *luxAB* marker genes were stably incorporated onto the CR30RNSLL (pADPTel) chromosome by a single integration event, and confirmed by Southern blot analysis. The *lacZY* genes allow the breakdown of lactose and lactose analogs. In culture media containing lactose or lactose analogues as the sole carbon source, as well as other additives, the *lacZY* genes are used as phenotypic markers to give the notified organism a characteristic colour. The lac operon is a naturally occurring metabolic system in bacteria. Godbout *et al*. (1995) have found that the *lac-lux* chromosomal marker system in other bacteria is stable and does not alter the growth or physiology of the host organism. The *lac-lux* marker system confers no selective advantage and it is not expected to be transferred to other bacteria due to the rarity of transfer mechanisms, such as homologous recombination or transposition. Introduction of these genes is expected to pose low hazard. The *luxAB* genes render bacteria bioluminescent, therefore functioning as a marker. There is no intrinsic hazard associated with these genes.

Exposure Considerations:

In general, it is expected that population levels of micro-organisms introduced into non-sterile soils would decline over time (Van Veen *et al.,* 1997; Winstanely *et al.,* 1993). Survival of *P. putida* is better in plant rhizosphere than in bulk soil, presumably because the plant provides the bacteria with exudates (OECD, 1997).

The aqueous solution (inoculum) containing *P. putida* CR30RNSLL(pADPTel) will be prepared in a contained facility in Carleton University, University of Saskatchewan, and the Biotechnology Research Institute of the National Research Council in Montreal that exceeds the Containment Level 1 requirements of Health Canada's Laboratory Biosafety Guidelines (1996). While *P. putida* is considered a risk group 1 organism, the laboratories in which the organism will be grown and the practices within these laboratories have met containment guidelines for level 2. The cells will be grown in liquid media, harvested, washed and resuspended in water at a concentration of 10^8 CFU/mL, and used within a few hours of preparation. The inoculum will be stored in closed polycarbonate bottles and transported to the field sites in a cooler.

According to Health Canada's Laboratory Biosafety Guidelines (1996), the recommended safe handling, storage, and transportation procedures for Risk Group 1 are as follows: appropriate personal protective equipment for handling the organism is recommended, including lab coat, closed-toe shoes and gloves. No special design features beyond those suitable for a well designed and functional laboratory with good microbiology practices are required. Recommended storage and transportation in an appropriately labeled container designed, constructed, filled, closed, secured and maintained is also required so that under normal conditions of transport, including handling, there will be no accidental release.

Approximately 1.25×10^{11} viable cells will be transported and applied on surface soil using a watering can to each field site annually for three years. The notifiers proposed to release the notified micro-organism at three closely monitored experimental field trial sites, one at Carleton University in Ottawa (10m x 4.7m), one at Eugene Lods Agricultural Research Facility on the Macdonald Campus of McGill University in St. Anne de Bellevue, Quebec (12m x 8m), and one at the Saskatchewan Wheat Pool Research Station located near the town of Watrous, Saskatchewan (15m x 10m). Each site will be divided into eight one-meter square plots and each will be inoculated with liquid culture.

Both the Carleton University and the McGill University sites are located on campus property near populated areas but are fenced for restricted access. The Carleton University site is located in the 100-year flood plain of the Rideau River approximately 35 metres from the high water level during the spring and 50 meters from the normal water level during the summer. The McGill University site is considered a research farmland. The terrain is almost level and located approximately 2 kilometers from the nearest water body, the St. Lawrence River. The third field study site is located in a non-populated area located near Watrous, Saskatchewan, 0.5 kilometers from the Saskatchewan Wheat Pool Research Station. The area is considered research and commercial farmland. The site is level and not directly adjacent to a water body. There are no endangered or threatened species known to live at any of the three experimental field study sites. The biological diversity at all three sites consists mostly of trees, grass, weeds and shrubs as well as insects and small mammals typical of a semi-urban ecosystem.

P. putida is not considered a human pathogen and it is unlikely that the modified strain would act differently. The small-scale field studies will be performed under controlled conditions where human exposure is limited to the personnel applying and sampling the inoculum *in situ*. Therefore, the notified micro-organism is deemed to pose a low risk to the general population.

Survival and Persistence

P. putida does not produce spores. Many biotic and abiotic factors, such as moisture, predators, soil composition, root growth, nutrients availability, temperature, influence bacterial survival in soils (van Veen *et al.,* 1997; OECD, 1997).

The notifiers presented data, from a previous microcosm validation study (Gagliardi *et al., 2*001), using soil samples taken from each of the three proposed field trial plots inoculated with *P. putida* CR30 and CR30RNSLL(pADPTel). The notifiers reported that the population of the notified strain survived in sterile bulk soil over a 28-day incubation period but exhibited a decrease in population density of two orders of magnitude. When inoculated into the rhizosphere of canola, the population density declined initially one order of magnitude during the first 28 days and then the population stabilized at this level over the next 42 days. This data shows that in non-sterile soils, survival of the notified strain was much better in the rhizosphere of soil cores planted with canola than in unplanted soil cores.

The notified strain could be recovered, by the addition of atrazine and nutrients, only from dried soil that had been associated with roots of harvested canola. In the absence of selection for atrazine degradation in soil, the levels of the notified strain declined to undetectable levels in bulk soil and rhizosphere in 165 days (i.e. less than approximately 100 viable cells per gram of soil). This is consistent with the decline in the populations of *P. putida* to below the detection limit (10^2 cfu/gm of root) over 105 days found by Glandorf *et al*. (1998), van Veen *at al*. (1997), and Winstanely *et al.* (1993).

The fate of the notified micro-organism, including over wintering survival, as well as its ability to transfer genes for atrazine degradation and tellurite resistance to indigenous soil bacteria will be determined during the course of the field trial study. It is expected that the *P. putida* CR30RNSLL (pADPTel) population in canola rhizosphere will decrease to undetectable levels as the atrazine is degraded or leached out of the soil, but may be recovered upon application of atrazine in the first few years. It is anticipated that over time the indigenous soil micro-organisms will displace the population of the introduced strain. Indeed, the first objective of this study is to predict the survival of the organism in the natural field soil, and to assess the validity of the laboratory microcosm study.

Dispersal

The field plots will be inoculated after May to reduce dispersal associated with spring run-off. Six-inch plastic borders will be placed around each 1 cubic metre plot to prevent surface run-off. These plastic borders will be inserted to extend three inches above the surface of the ground. This type of barrier has previously been reported to be effective in preventing dispersal of *P. putida* (Molina *et al*., 1998). In a previous field release experiment involving a different micro-organism at these three sites, the notifier detected no off-site dispersal of that organism immediately outside the plots. Since all the three experimental sites are level or nearly level, the potential for dispersal of the notified strain in the surrounding area by run-off is considered low. In the event of off-site dispersal, the notifiers plan to excavate the soil to a depth of 15 centimetres and transport it to the laboratory for autoclaving.

The field trial plots and surrounding areas will be closely monitored to detect adverse ecological effects. The area surrounding the field trial plots will be monitored monthly to detect off-site dispersal at four compass points located a distance of four meters from the perimeter of the plots. A fifth point at the Carleton University site closest to the Rideau River will be monitored. Contingency plans are in place in case of inadvertent release. Thus, the environmental exposure of the notified strain outside the three field trial sites is likely to be low.

Depending on the soil type, the capacity for bacteria to move laterally through percolation or active bacterial transport is limited to a few centimetres in bulk soil (OECD, 1997; Smit *et al.,* 1992. However, in the rhizosphere, the growth of roots assists in distributing bacteria laterally

through the soil. Bacteria can be dispersed from the site of application by wind, by fauna existing at the site or by run-off with surface water (Gillespie *et al.,* 1995). In this trial, aerosol generation and wind dispersal of the notified strain will be limited by applying it using a watering can. Hence, no significant dispersal of the notified strain is expected outside the field trial sites.

Gene Transfer

P. putida has the capacity for gene transfer by means of transformation, transduction, and conjugation. Conjugation between pseudomonads has been detected in aquatic and soil environments (OECD, 1997). Transfer frequencies have been found to be influenced by factors such as soil moisture, temperature, and the presence of physical substrates which will allow for survival and/or gene transfer (OECD, 1997).

Indigenous rhizosphere and soil bacteria capable of accepting and expressing the recombinant pADPTel plasmid are expected to acquire the tolerance to tellurite and to degrade atrazine. Gene transfer is expected to occur within the three field trial plots in the presence of atrazine, but it is expected to decline as atrazine is degraded or leached out of the soil. Selective pressure resulting from the addition of a small amount of atrazine is expected to be transient and localized within the field plots. Indeed, the second objective of this study is to evaluate the transfer and persistence of these genes, in order to develop a method that can predict the potential for and the frequency of gene transfer between other soil microorganisms in microcosms and field soils.

Ampicillin, chloramphenicol, and tetracycline resistance was passed on to the notified strain from the parent strain isolated in the natural environment. These resistance genes could be plasmid-encoded, but the design of the field trial will not provide a selective advantage to micro-organisms with resistance to these antibiotics. There is not expected to be transfer of these genes off the field trial site. Because the naturally occurring antibiotic resistance genes for naladixic acid, streptomycin and rifampicin are believed to be chromosomally encoded, horizontal gene transfer of these resistance genes to the environment is not expected to be significant.

The likelihood of horizontal gene transfer of the pADPTel plasmid to plants and animals is low. For gene transfer to be successful, a number of events must occur sequentially, the likelihood of which depend on the availability of the plasmid, and the ability of the host organism to take up and transform the foreign prokaryotic DNA. *Agrobacterium*-mediated transformation is possible, but would be exceedingly rare in this case. The inserted genetic materials would unlikely pose any harm to plants and animals in the field trial sites and surrounding areas.

Approved by:

_____ _____

Director, New Substances Branch
Risk Assessment Directorate
Environment Canada

Date

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