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Generation and initial characterization of *Pseudomonas stutzeri* KC mutants with impaired ability to degrade carbon tetrachloride

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Abstract Under iron-limiting conditions, *Pseudomonas stutzeri* KC secretes a small but as yet unidentified factor that transforms carbon tetrachloride (CT) to CO₂ and non-volatile products when activated by reduction at cell membranes. *Pseudomonas fluorescens* and other cell types activate the factor. Triparental mating was used to generate kanamycin-resistant *lux::Tn5* recombinants of strain KC. Recombinants were streaked onto the surface of agar medium plugs in microtiter plates and were then screened for carbon tetrachloride degradation by exposing the plates to gaseous ¹⁴C-carbon tetrachloride. CT⁺ recombinants generated nonvolatile ¹⁴C-labeled products, but four CT⁻ recombinants did not generate significant nonvolatile ¹⁴C-labeled products and had lost the ability to degrade carbon tetrachloride. When colonies of *P. fluorescens* were grown next to colonies of CT⁺ recombinants and were exposed to gaseous ¹⁴C-carbon tetrachloride, ¹⁴C-labeled products accumulated around the *P. fluorescens* colonies, indicating that the factor secreted by CT⁺ colonies had diffused through the agar and become activated. When *P. fluorescens* was grown next to CT⁻ colonies, little carbon tetrachloride transformation was observed, indicating a lack of active factor. Expression of *lux* reporter genes in three of the CT⁻ mutants was regulated by added iron and was induced under the same iron-

limiting conditions that induce carbon tetrachloride transformation in the wild-type.

Key words Transposon mutagenesis · Carbon tetrachloride · Biotransformation · Biodegradation · Luciferase · *Pseudomonas stutzeri* KC · Reporter genes · Mutants

Abbreviations CT Carbon tetrachloride · Km Kanamycin · LB Luria broth · RLU Relative light units · Rf Rifampicin · TSB Tryptic soy broth

Introduction

Carbon tetrachloride (CT) is a suspected carcinogen that also causes acute liver toxicity in animals (Sittig 1985). Its production has been banned under the terms of the amended Montreal protocol because it is an ozone-depleting agent (United Nations Environmental Programme 1994). Most denitrifying organisms that degrade carbon tetrachloride do so slowly with the accumulation of chloroform, a compound that can be even more persistent than carbon tetrachloride (Egli et al. 1988; Semprini et al. 1992).

Pseudomonas stutzeri strain KC is an aquifer isolate that transforms carbon tetrachloride to carbon dioxide (Criddle et al. 1990; Lewis and Crawford 1993; Tatara et al. 1993), formate (Dybas et al. 1995), and other non-volatile products without the formation of chloroform (Criddle et al. 1990; Lewis and Crawford 1993; Tatara et al. 1993). Rapid carbon tetrachloride transformation requires a small (500 Da) factor secreted by exponential-stage strain KC cells grown under Fe³⁺-limiting conditions, together with actively growing cells capable of regenerating the secreted factor. The transformation occurs only under anoxic conditions, although the factor is produced under both oxic and denitrifying conditions (Dybas et al. 1995). Organisms that usually do not degrade carbon tetrachloride are able to do so when the factor is provided (Tatara et al. 1995). Of special interest are the genes re-

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quired for production, secretion, and activation of the factor. To identify these genes, methods are needed for rapid and efficient screening of large numbers of mutants.

Most of the microorganisms that degrade volatile, halogenated hydrocarbons produce CO₂ and nonvolatile products (Jain and Criddle 1995). The nonvolatile products accumulate or are utilized for growth (Nielsen 1990; Chaudhry and Chapalamadugu 1991; Fetzner and Lingens 1994; Jain and Criddle 1995). When ¹⁴C-labeled hydrocarbons are degraded, nonvolatile ¹⁴C-labeled products are often generated (Wackett and Gibson 1983; Lewis and Crawford 1993; Sepúlveda et al. 1997). To obtain mutants with impaired ability to degrade carbon tetrachloride, we screened a large number of *P. stutzeri* KC *lux::Tn5* mutants for failure to produce nonvolatile ¹⁴C-labeled products from ¹⁴C-carbon tetrachloride. Mutants were then characterized for *lux* expression by assaying luciferase under different growth conditions.

Materials and methods

Strains and plasmids

P. stutzeri strain KC (DSM 7136, ATCC 55595), isolated from an aquifer in Seal Beach (Calif., USA) (Criddle et al. 1990), was maintained on nutrient agar. *Pseudomonas fluorescens* (ATCC 13525) was obtained from the Department of Microbiology, Michigan State University. Rifampicin-resistant (Rf^R) *P. stutzeri* KC strains were selected in our laboratory on nutrient agar plates containing rifampicin (100 µg/ml) from a set of spontaneous Rf^R strain KC mutants. An Rf^R strain KC isolate was used in the triparental matings described below in order to counterselect for the *Escherichia coli* donor and helper strains. One Rf^R strain, KC137, was selected for subsequent studies because its carbon tetrachloride degradation capability matched that of the wild-type. All *Pseudomonas* strains were grown at 20–25°C with constant shaking (150 rpm). *E. coli* DH5α containing pRL1063a, a kanamycin-resistant (Km^R) transposon delivery plasmid for a *luxAB::Tn5* construct (Wolk et al. 1991), and *E. coli* containing the Km^R helper plasmid pRK2013 (Ditta et al. 1980) were grown in Luria Broth medium (Sambrook et al. 1989) with 35 µg Km/ml at 37°C and with shaking at 200 rpm.

Chemicals and media

Nonradioactive carbon tetrachloride (99% pure) was purchased from Aldrich Chemical (Milwaukee, Wis., USA). ¹⁴C-carbon tetrachloride (> 99% pure; 250 µCi, specific activity of 4.3 mCi/mmol) purchased from NEN Dupont Research Products (Boston, Mass., USA) was dissolved in iso-octane to a concentration of 0.136 µmol/µl (1.4 µCi/µl) and was stored at –20°C.

Precipitate-free, iron-free Simulated Groundwater Medium (SGM) was prepared as described by Dybas et al. (1995) with some modifications. The modified medium used half of the sodium hydroxide and potassium phosphate specified in the original procedure, and Fe-free TN2 trace element solution (Criddle et al. 1990) instead of SGM trace element solution. The medium was prepared in acid-washed glassware, adjusted to an initial pH of 8.2, autoclaved at 121°C for 25 min, transferred to a laboratory bench for quiescent settling of precipitate, and decanted after 24 h. The precipitate-free decanted medium was reautoclaved for 25 min at 121°C and cooled before use. Precipitate-free SGM contained 23 mM acetate, 19 mM nitrate, and 0.1 M phosphate as determined using a Dionex model 2000i-SP ion-chromatography system (Sunnyvale, Calif., USA). No iron was detected using a Perkin Elmer (Norwalk, Conn., USA) model 3110 graphite furnace atomic absorption spectroscopy system.

Anoxic medium D adjusted to an initial pH of 8.0 was prepared in acid-washed glassware as previously described (Criddle et al. 1990). Noble agar, nutrient agar, nutrient broth, and tryptic soy broth (TSB) were obtained from Difco (Detroit, Mich., USA).

Transposon mutagenesis

Triparental matings (Simon et al. 1983) were used to mobilize plasmid pRL1063a into Rf^R *P. stutzeri* KC137. *E. coli* DH5α containing the donor plasmid pRL1063a and *E. coli* DH5α containing the helper plasmid pRK2013 were grown overnight in LB medium supplemented with 35 µg Km/ml at 37°C and with shaking at 200 rpm. These cultures were transferred to fresh LB without antibiotics using a 4% (by vol.) inoculum and were then grown at the same temperature and shaking speed for 4 h (final cell concentration, ~10⁹ cfu/ml). Cultures of the recipient strain KC137 were grown under aerobic conditions for 16 h in TSB or LB containing 35 µg Rf/ml, or under denitrifying conditions for 2 days in medium D containing 35 µg Rf/ml (final cell concentration, ~10⁹ cfu/ml). All cultures were washed twice and then resuspended to the original volume using sterile 0.9% NaCl solution. Mixes were prepared by combining 100 µl of *E. coli* DH5α (pRL1063), 100 µl of *E. coli* DH5α (pRK2013), and 500 µl of strain KC137 in sterile microcentrifuge tubes. Individual strains and 100 µl/100 µl pairwise mixes were included as controls to ensure that Km^R/Rf^R double mutants were not present in the mating plates. Fifty microliters of each mixture was spotted on 13-mm filters (containing pores of 0.45 µm in diameter; Millipore, Bedford, Mass., USA) on nutrient agar plates. The plates were incubated at 37°C for 10 h and then at room temperature for 10 h. The filters were transferred to microcentrifuge tubes containing 1 ml of a sterile 0.9% NaCl solution, and the bacteria grown on the filter were resuspended in the buffer. Resuspended cells were distributed in 200-µl aliquots onto nutrient agar plates containing 50 µg Km/ml and 50 µg Rf/ml and were then incubated at room temperature for 3 days.

Microtiter plate assay for ¹⁴C-carbon tetrachloride degradation

Sterile microtiter plates (96 wells) were filled with 200 µl of medium D (pH 8) containing 15 g Noble agar l⁻¹ and 25 µg Km/ml. Recombinants obtained by triparental mating were streaked on the agar surfaces of the microtiter plate wells using sterile toothpicks. Inoculated microtiter plates were transferred to a 3.8-l steel paint can (Freund Can Company, Chicago, Ill., USA) containing clean glass marbles to minimize the gas volume within the can (working vol. ≈ 1.9 l). Air in the can was replaced by a 95% nitrogen/5% hydrogen mixture by passing the open can through the interlock of an anaerobic glove box (Coy Laboratories, Detroit, Mich., USA). A sealed 12 × 32-mm glass vial containing 1.4 µmol (14 µCi) of ¹⁴C-carbon tetrachloride was attached to the inner wall of the container. The septum of the vial was punctured with a needle, releasing ¹⁴C-carbon tetrachloride into the can, and the can was immediately sealed and removed from the glove box for incubation. Plates inside the steel can were exposed to vapor phase ¹⁴C-carbon tetrachloride for 5 days at 20°C and then were vented overnight in a chemical hood. Agar plugs from the microtiter plates were transferred to 10 ml of Safety-solve scintillation cocktail (RPI, Prospect, Ill., USA) and were assayed for nonvolatile radioactivity for 3 min with a 1500 Tri-carb liquid scintillation counter (Packard Instrument, Downers Grove, Ill., USA).

The recombinants that did not produce significant ¹⁴C-labeled nonvolatile products in the microtiter plate assay were analyzed by gas chromatography (GC) to confirm loss of carbon tetrachloride degradation capabilities. Recombinants were grown overnight in 5 ml TSB containing 35 µg Km/ml. These cultures were used as a 1% inoculum (by vol.) for 10 ml of anoxic medium D (pH 8) vials containing 35 µg Km/ml and 350 ng of sterile carbon tetrachloride. Cultures were incubated upside down for a week under anoxic conditions at 20°C and with shaking at 150 rpm. The carbon tetrachloride remaining in the vials was assayed by GC as described by Tataru et al. (1993).

Table 1 Results of the ¹⁴C microtiter plate assay and the gas chromatography assay

Strain	dpm per well in microtiter plate assay	% CT degraded after 1 week in gas chromatography assay ^c
Noninoculated controls	82 ± 15 ^a	9 ± 7
Wild-type <i>Pseudomonas stutzeri</i> KC	2,218 ± 437 ^b	100 ± 0
Recombinants capable of degrading CT in both assays		
KC300	1,416	100 ± 0
Recombinants with impaired ability to degrade CT in both assays		
KC657	465	28 ± 26
KC1896	511	18 ± 8
KC2753	291	28 ± 8
KC3164	459	21 ± 13

^a Average ± one standard deviation for seven replicates

^b Average ± one standard deviation for nine replicates

^c Average ± one standard deviation for triplicates

Petri plate assay for secreted factor production

By itself, the secreted factor produced by strain KC does not reliably transform carbon tetrachloride; however, reliable transformation is observed when it is combined with viable whole cells (Dybas et al. 1995). Tatara (1996) has demonstrated that carbon tetrachloride transformation also occurs when the secreted factor is added to crude cell membrane preparations supplemented with NADH. Decreased transformation rates were observed when the factor was added to crude cell membranes lacking NADH, with little or no transformation when the secreted factor was added to cytoplasmic fractions. Both cell membranes and NADH were required for maximum activation. Of interest is the fact that *P. fluorescens* and many other cell types can mediate activation of the secreted factor (Tatara et al. 1995). In order to determine if the CT⁻ mutants were impaired for secreted factor production, secretion, or activation, CT⁻ mutants and cells of *P. fluorescens* were grown on Petri plates containing solid medium D (pH 8). Colonies of CT⁻ mutants and *P. fluorescens* were grown on Petri plates in an alternating "checkerboard" layout with each colony separated by 1.5 cm from adjacent colonies of the other organism. Petri plates were incubated following the same protocol used for the microtiter plates. After the incubation period, 1-cm² agar squares including and surrounding each colony were transferred to 10 ml scintillation cocktail and were counted by the liquid scintillation counter.

Expression of *lux* genes in strain KC mutants

CT⁻ and selected CT⁺ recombinants were grown in 5 ml TSB containing 35 µg Km/ml for 24 h at 25 °C and with shaking at 150 rpm. These cultures were used as 0.5% inocula (v/v) for 25-ml cultures of TSB or Fe-free, precipitate-free SGM containing 35 µg Km/ml supplemented with 0, 5, 10, or 20 µM Fe³⁺ as FeNH₄(SO₄)₂ · 12H₂O. Cultures were grown for 29 h at 25 °C and with shaking at 150 rpm, and then were used as 0.5% inocula (v/v) for 100-ml cultures containing the same antibiotic and Fe³⁺ concentrations. Growth of these cultures was monitored over a 48-h period by periodically removing 1-ml aliquots and measuring optical density at 600 nm.

The light emission assay used to detect luciferase activity was performed on a Berthold Lumat LB 9501 luminometer (EG&G Wallac, Gaithersburg, Md., USA) by combining 5-µl culture aliquots with 50 µl of a solution containing 20 mg bovine serum albumin/ml and 1 µl N-decyl aldehyde/ml. Samples were vortexed for 30 s and assayed for light emission (relative light units) for 1 min.

Results

Generation of mutants with impaired ability to degrade carbon tetrachloride

Three thousand five hundred recombinants were obtained from 44 independent triparental mating events. Most re-

combinants accumulated roughly the same level of ¹⁴C-labeled nonvolatile products in the agar plugs as wild-type cells (1,500–3,000 dpm). Only 38 recombinants accumulated fewer than 1,000 dpm: 4 were auxotrophs that could not grow in the defined medium used for the GC assay; 30 grew poorly in solid medium D, but grew and degraded carbon tetrachloride in liquid medium; and 4 were classified as putative CT⁻ mutants based on their growth in liquid and solid media and on their inability to degrade significant CT in either assay. A PCR probe specific to strain KC was used to confirm that the putative CT⁻ mutants were strain KC cells (Dybas et al. 1998). Table 1 summarizes results for noninoculated controls, the wild-type, one of the recombinants that retained the ability to degrade carbon tetrachloride (strain KC300), and the four mutants that failed to transform carbon tetrachloride in either assay (CT⁻ mutants). Although the CT⁻ mutants were only tested once in the microtiter plate assay, the results of the GC assay provided statistically significant proof that these mutants were impaired in carbon tetrachloride degradation.

Table 2 Petri plate assay of secreted factor production by wild-type and recombinant KC colonies grown in the presence of *Pseudomonas fluorescens* and ¹⁴C- carbon tetrachloride. *P. fluorescens* was grown alone or in an alternating checkerboard configuration with wild-type KC, KC300, KC657, KC1896, KC2753, or KC3164. All colonies were separated by 1.5 cm. Radioactivity is reported as disintegrations per minute per square centimeter of agar for eight replicates

Incubation condition	dpm/cm ² (avg ± sd)
No-cell control	42 ± 5
<i>P. fluorescens</i> alone	198 ± 35
<i>P. fluorescens</i> +wild-type strain KC	1,583 ± 335
<i>P. fluorescens</i> +autoclaved wild-type strain KC	215 ± 32
<i>P. fluorescens</i> +KC300 (CT ⁺)	1,905 ± 179
<i>P. fluorescens</i> +KC657 (CT ⁻)	95 ± 13
<i>P. fluorescens</i> +KC1896 (CT ⁻)	84 ± 13
<i>P. fluorescens</i> +KC2753 (CT ⁻)	114 ± 9
<i>P. fluorescens</i> +KC3164 (CT ⁻)	102 ± 14

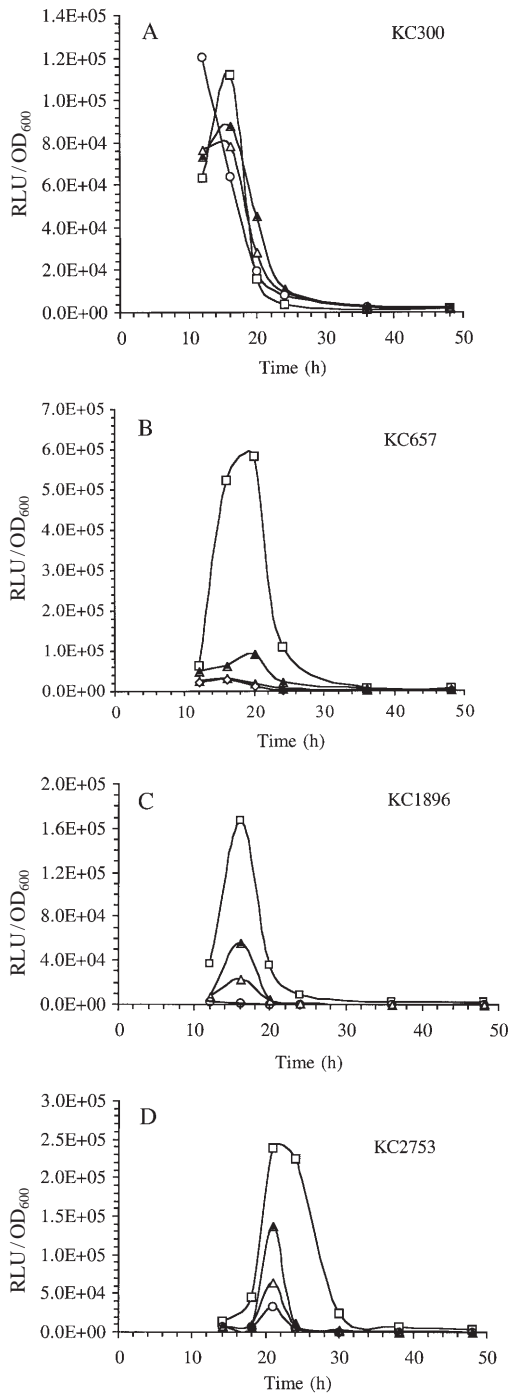


Fig. 1 Luciferase activity, expressed as relative light units divided by OD₆₀₀, during growth of **A** strain KC 300, **B** strain KC 657, **C** strain KC 1896, and **D** strain KC 2753 in Simulated Groundwater Medium containing 1 \square , 5 \blacktriangle , 10 \triangle , and 20 \circ μM Fe³⁺. Due to the low values of OD₆₀₀ prior to 12 h of growth and in 0 μM Fe³⁺, this ratio was only computed for samples after 12 h of growth and for Fe³⁺ concentrations > 0 μM . Plotted values are the average of two independently grown cultures. Data is not shown for strain KC 3164, which exhibited very low *lux* expression and no clear pattern with increasing iron concentration (similar to strain KC 300)

Evidence that the CT⁻ mutants were impaired in secreted factor production

Table 2 summarizes the results of experiments using an agar-based assay to detect secreted factor production. When *P. fluorescens* was grown in the presence of wild-type strain KC or strain KC *lux*: :Tn5 recombinants capable of degrading carbon tetrachloride (such as strain KC300), the radioactivity that accumulated in the agar plugs around the *P. fluorescens* colonies was more than sevenfold the amount that accumulated when *P. fluorescens* was grown alone (Table 2). When *P. fluorescens* was grown in the presence of any of the putative CT⁻ mutants, the nonvolatile radioactivity was only twice that of noninoculated controls. This indicates that the four putative CT⁻ mutants either do not produce significant levels of CT-degrading factor or secrete a largely inactive form of it.

Expression of *lux* genes in CT⁻ mutants

There was no significant difference between the time course for growth of *lux*: :Tn5 recombinants that were impaired in carbon tetrachloride degradation (e.g., strain KC657) and the time course for growth of strain KC300 and many other recombinants that retained carbon tetrachloride degradation activity. The maximum optical density was 0.8 for SGM cultures and 1.6 for TSB cultures after 20 h (data not shown). However, for all of the recombinants except strain KC3164, addition of Fe³⁺ resulted in dramatically reduced luciferase activity (Fig. 1). This effect was not observed with CT⁺ recombinants such as strain KC300 (Fig. 1A). Complex media supported low levels of *lux* expression in the recombinants (Fig. 2), but defined mineral medium supported high levels of expression in three of the four CT⁻ mutants (Fig. 1B–D). For cells grown in SGM containing only 1 μM Fe³⁺, maximal *lux* expression was sevenfold the level achieved by cells grown in TSB. In contrast, the CT⁺ recombinant strain

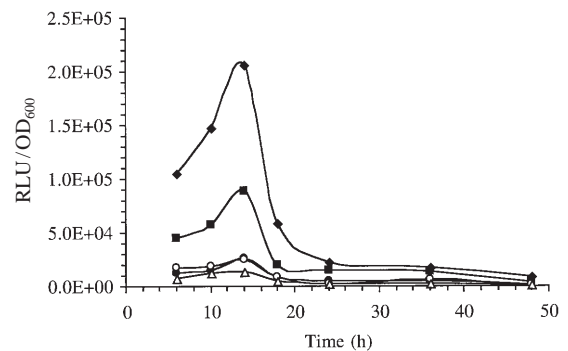


Fig. 2 Luciferase activity, expressed as relative light units divided by OD₆₀₀, during growth of strain KC 300 \blacklozenge , strain KC 657 \blacksquare , strain KC 1896 \bullet , strain KC 2753 \circ , and strain KC 3164 \triangle in tryptic soy broth. Plotted values are the average of two independently grown cultures

KC300 had strong *lux* expression in SGM regardless of Fe^{3+} concentration (Fig. 1 A), and expression in TSB was only 1.7-fold the level achieved during growth in SGM.

Discussion

The ^{14}C -carbon tetrachloride assay was a reliable and efficient means of rapidly screening large numbers of recombinants for carbon tetrachloride degradation. GC analysis confirmed that four mutants were impaired in carbon tetrachloride degradation. GC confirmation was important because some recombinants grew poorly on agar, giving artificially low counts. It is also important because a mutant might degrade carbon tetrachloride without generating nonvolatile products, and such a mutant would be incorrectly classified as CT^- .

An interesting result was the finding that the factor secreted by strain KC could diffuse through agar and still transform carbon tetrachloride when "activated" by viable whole cells such as colonies of *P. fluorescens* (Table 2). Elevated levels of nonvolatile ^{14}C -labeled products were obtained for *P. fluorescens* colonies adjacent to colonies of wild-type strains KC or KC300. Low levels of nonvolatile ^{14}C -labeled products were obtained for *P. fluorescens* colonies adjacent to colonies of CT^- mutants (strains KC657, KC1896, KC2753, and KC 3164). This result indicates that the mutants were defective in production and/or secretion of the secreted factor.

The *lux*: :Tn5 construct on pRL1063a generates a promoterless *lux* transcriptional fusion. Therefore, *lux* is expressed only when the native promoter of the interrupted gene is activated. For three of the four CT^- mutants (strains KC657, KC1896, and KC2753), *lux* expression studies confirmed that the genes required for carbon tetrachloride degradation are expressed under Fe^{3+} -limiting conditions in the late exponential phase of growth (Fig. 1). Mutant KC3164 did not show any significant luciferase expression because the transposon inserted in the wrong orientation with respect to the native promoter, as indicated by subsequent DNA sequence analysis (data not shown). The Fe^{3+} -dependent response of the CT^- transformants was not an artifact introduced by the transposon. CT^+ mutant KC300 did not show significant differences in *lux* expression when grown at different iron concentrations (Figs. 1 A, 2), presumably because the transposon inserted itself in a gene that is not regulated by Fe^{3+} availability. For the putative CT^- mutants, light emission was greater for cultures grown in defined mineral medium SGM (Fig. 1) than for cultures grown in complex medium TSB (Fig. 2). In contrast, light emission for the CT^+ mutant KC300 was greater in complex media (Figs. 1 A, 2). Further details related to the identity of the genes interrupted in the CT^- strains will be provided in a separate publication.

The conditions that induce luciferase expression in the CT^- strains correspond to conditions that induce secreted factor production in the wild-type strain (Tatara 1996). Expression of the *lux* genes was inversely correlated to

the amount of Fe^{3+} added for CT^- mutants (Fig. 1). This pattern was consistent with previous reports indicating that Fe^{3+} addition stimulated cell growth but reduced carbon tetrachloride degradation (Criddle et al. 1990; Tatara et al. 1995).

This is the first report of mutants with impaired ability to degrade carbon tetrachloride. Initial characterization indicates that these mutants either do not produce the carbon-tetrachloride-degrading factor or they produce a non-functional form of it. The observed interaction of the secreted factor with other cell types and its apparent ability to obtain reducing equivalents thereby suggest a possible role in cell/cell communication. On the other hand, its known regulation by ferric iron and cobalt suggests a possible role in trace metal acquisition, perhaps not unlike that recently reported for the cytochrome secreted by *Geobacter sulfurreducens* (Seeliger et al. 1998). Further studies are under way to characterize the genes inactivated by the *lux*: :Tn5 transposon in the strain KC mutants. Sequencing and identification of the interrupted genes is expected to provide insight into the identity and physiological role of the carbon-tetrachloride-transforming secreted factor and its mechanism of transformation.

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References

- Chaudhry GR, Chapalamadugu S (1991) Biodegradation of halogenated organic compounds. *Microbiol Rev* 55:59–79
- Criddle CS, DeWitt JT, Grbić-Galić D, McCarty PL (1990) Transformation of carbon tetrachloride by *Pseudomonas* sp. strain KC under denitrification conditions. *Appl Environ Microbiol* 56:3240–3246
- Ditta G, Stanfield S, Corbin D, Helinski DR (1980) Broad host range cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. *Proc Natl Acad Sci USA* 77:7347–7351
- Dybas MJ, Tatara GM, Criddle CS (1995) Localization and characterization of the carbon tetrachloride transformation activity of *Pseudomonas* sp strain KC. *Appl Environ Microbiol* 61:758–762
- Dybas MJ, et al (1998) Pilot-scale evaluation of bioaugmentation for *in-situ* remediation of a carbon tetrachloride-contaminated aquifer. *Environ Sci Technol* 32:3598–3611
- Egli C., Tschan T, Scholtz R, Cook AM, Leisinger T (1988) Transformation of tetrachloromethane to dichloromethane and carbon dioxide by *Acetobacterium woodii*. *Appl Environ Microbiol* 54:2819–2823
- Fetzner S, Lingens F (1994) Bacterial dehalogenases: biochemistry, genetics, and biotechnological applications. *Microbiol Rev* 58:641–685
- Jain M, Criddle CS (1995) Metabolism and cometabolism of halogenated C-1 and C-2 hydrocarbons. In: Singh VP (ed) *Bio-transformations: microbial transformations of health risk compounds*. Elsevier, Amsterdam, The Netherlands, pp 65–102

- Lewis TA, Crawford RL (1993) Physiological factors affecting carbon tetrachloride dehalogenation by the denitrifying bacterium *Pseudomonas* sp. strain KC. *Appl Environ Microbiol* 59: 1635–1641
- Lewis TA, Crawford RL (1995) Transformation of carbon tetrachloride via sulfur and oxygen substitution by *Pseudomonas* sp. strain KC. *J Bacteriol* 177: 2204–2208
- Nielsen AH (1990) The biodegradation of halogenated organic compounds. *J Appl Bacteriol* 69: 445–470
- Sambrook J, Fritsch EL, Maniatis T (1989) *Molecular cloning. A laboratory manual*, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- Seeliger S, Cord-Ruwisch R, Schink B (1998) A periplasmic and extracellular c-type cytochrome of *Geobacter sulfurreducens* acts as a ferric ion reductase and as an electron carrier to other acceptors or to partner bacteria. *J Bacteriol* 180: 3686–3691
- Semprini L, Hopkins GD, McCarty PL, Roberts PV (1992) In-situ transformation of carbon tetrachloride and other halogenated compounds resulting from biostimulation under anoxic conditions. *Environ Sci Technol* 26: 2454–2461
- Sepúlveda-Torres L del C, Dybas MJ, Criddle CS (1997) Solid phase bioremediation of carbon tetrachloride by *Pseudomonas stutzeri* strain KC. In: Alleman BC, Leeson A (eds) *The Fourth International in-situ and On-site Bioremediation Symposium*. Battle Press, Columbus, pp 33–37
- Simon R, Priefer U, Pühler A (1983) A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram-negative bacteria. *Biotechnology* 1: 784–790
- Sittig M (1985) *Handbook of toxic and hazardous chemicals and carcinogens*, 2nd edn. Noyes, New York
- Tatara GM (1996) PhD Thesis, Michigan State University, MI, USA
- Tatara GM, Dybas MJ, Criddle CS (1993) Effects of medium and trace metals on kinetics of carbon tetrachloride transformation by *Pseudomonas* sp. strain KC. *Appl Environ Microbiol* 59: 2126–2131
- Tatara GM, Dybas MJ, Criddle CS (1995) Biofactor-mediated transformation of carbon tetrachloride by diverse cell types. In: Hinchee RE, Leeson A, Semprini L (eds) *Bioremediation of chlorinated solvents*. Battle Press, Columbus, pp 69–76
- United Nations Environment Programme (1994) Adjustment to the Montreal protocol on substances that deplete the ozone layer. In: Sands P, Tarasofsky R, Weiss M (eds) *Principles of international environmental law*. 2A. Documents in international environmental law. Manchester University Press, Manchester, pp 208–223
- Wackett LP, Gibson DT (1983) Expression of naphthalene oxidation in *Escherichia coli* results in the biosynthesis of indigo. *Science* 222: 167–169
- Wolk CP, Cai Y, Panoff JM (1991) Use of a transposon with luciferase as a reporter to identify environmental responsive genes in a cyanobacterium. *Proc Natl Acad Sci USA* 88: 5355–5359