Niche Adjustment for Bioaugmentation with *Pseudomonas* sp. strain KC

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ABSTRACT

To be effective, novel organisms introduced into the environment must be able to survive and compete with indigenous organisms. However, to minimize the possibility of ecological disturbance, colonization should ideally be constrained. A possible solution is to create a temporary niche for the introduced organism. Alkalinity addition creates just such a niche, by reducing the bioavailability of essential trace metals, such as iron, thereby favoring organisms with efficient trace metal scavenging systems. We evaluated alkaline pH adjustment with *Pseudomonas* sp. strain KC, a denitrifying aquifer organism that degrades carbon tetrachloride (CT) under denitrifying conditions. We compared the kinetic parameters of strain KC with those of its potential competitors (other denitrifiers) in groundwater from a CT-contaminated aquifer at Schoolcraft, Michigan. Under moderately alkaline conditions (7.9 to 8.2), strain KC had a higher maximum specific growth rate and yield. With alkaline adjustment, strain KC grew and degraded CT in columns containing aquifer solids from the Schoolcraft site and in slurries of aquifer material from Hanford, Washington. Upon reducing pH, a rapid decline in the KC population followed.

INTRODUCTION

Pseudomonas sp. strain KC rapidly degrades carbon tetrachloride (CT), without production of chloroform under denitrifying conditions. The primary degradation product is carbon dioxide. Stimulation of indigenous denitrifying communities usually results in slow degradation of CT and production of chloroform. Thus, bioaugmentation with strain KC offers pathway and kinetic advantages, provided strain KC can compete. We evaluated the possibility that alkaline pH adjustment would enhance the competitiveness of strain KC. For this organism, the competitive advantage of alkaline niche adjustment is due to changes in the speciation of iron and copper. Ferric iron is least soluble in the pH range 8.0 to 8.2 (Stumm et al. 1981). Adjustment of pH to levels within the alkaline range favors efficient iron scavengers. *Pseudomonas* sp. strain KC is an excellent competitor for iron, secreting siderophores of both the hydroxymate and catechol type to sequester iron for uptake (Dybas et al. 1995). Strain KC also secretes other factors under iron-limiting conditions, most significantly, a low molecular weight factor that fortuitously degrades CT (Dybas et al. 1995).

Copper speciation also has ecological significance for strain KC; at neutral pH, trace copper inhibits growth (Criddle et al. 1990), but, at moderately alkaline levels, trace copper does not inhibit growth and is actually required for CT degradation (Tatara et al. 1993). These observations suggest that pH can be used to enable and confine strain KC colonization. Alkaline pH favors growth; but when the pH drops to near neutral levels, trace copper becomes inhibitory and iron availability increases, diminishing the competitive advantage accrued from efficient iron scavenging.

In this paper, we provide evidence that pH can be used to control the competitiveness and persistence of Pseudomonas sp. strain KC in bioaugmentation applications.

EXPERIMENTAL PROCEDURES AND MATERIALS

Chemicals

Carbon tetrachloride (CT, 99% purity) was obtained from Aldrich Chemical Co., Milwaukee, Wisconsin. Thimersol was obtained from Sigma Chemical Co., St. Louis, Missouri. All chemicals used were ACS reagent grade (Aldrich or Sigma Chemical Co.). All water used in reagent preparation was deionized 18 Mohm resistance or greater.

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Media and Growth Conditions

Medium D was prepared and dispensed in 28-mL serum tubes or modified 1-L Wheaton bottles as previously described (Tatara et al. 1993). Nutrient broth and nutrient agar (Difco) plates were prepared according to manufacturer's instructions. The recipe for SGW (synthetic groundwater) medium is described in Tatara et al. (1995).

Groundwater and Aquifer Materials

Groundwater from a CT-contaminated aquifer in Schoolcraft, Michigan, was used in all batch and column studies. Aquifer material from the Schoolcraft site was provided courtesy of Brown and Root Environmental (Holt, Michigan). Aquifer material from Hanford, Washington, was provided by Battelle Pacific Northwest Laboratories.

Analytical Methods

CT was assayed by injection of headspace gas into a gas chromatograph equipped with an electron capture detector, as described previously (Tatara et al. 1993). Nitrate, nitrite, and acetate were assayed by ion chromatography.

Kinetic Characterization of Strain KC and Indigenous Organisms in Schoolcraft Groundwater

The kinetics of growth of strain KC and organisms indigenous to the Schoolcraft aquifer were determined in batch experiments. To determine the kinetics of growth of indigenous flora, unpasteurized Schoolcraft groundwater was supplemented with 30 mM acetate, 12 mM nitrate, 0.1 mM phosphate, and 10 g $NaHCO_3$/L to give an initial pH of 8.2. To determine the kinetics of growth of strain KC, strain KC was added to supplemented, pasteurized groundwater as a washed 1% inoculum from a culture grown aerobically for 72 hours in medium D. Pasteurization entailed heating the water at 65°C for 8 hours. Unlike other procedures (autoclaving, filter sterilization), pasteurization eliminated indigenous flora without adversely affecting the growth of strain KC.

The maximum specific growth rate μ_m was obtained for log growth phase cells using optical density measurements at a wavelength of 660 nm and the relationship: $\mu_m = [\ln(X_f/X_i)]/(t_f - t_i)$, where X_f and X_i represent the final and initial optical density, respectively, and t_f and t_i are the final and initial time, respectively. Yield on nitrate and nitrite for both cultures was calculated using dry weight measurements obtained by filtering a known volume through 0.2 μm filter membranes and washing the cells. The filter membranes were dried overnight at 104°C and weighed. The maximum specific rates of nitrate and nitrite utilization were calculated by dividing the instantaneous rates of nitrate or nitrite consumption by the dry weight concentration.

Persistence of Strain KC in Schoolcraft Groundwater

To evaluate long-term survival of strain KC in Schoolcraft groundwater, strain KC ($2x10^6$-$1x10^7$ cells/mL) was added to pasteurized Schoolcraft groundwater that had been adjusted to a pH of 8.2 using sterile 100 mM sodium carbonate solution and sterile CO_2 gas. Pasteurized groundwater was used to allow reliable enumeration of strain KC by plate counts. The water contained no added carbon source or electron acceptor. Samples were removed at 1- to 4-day intervals and assayed for strain KC by serial dilution and plate count on nutrient agar plates. After 14 days, the pH was adjusted to pH 7.5 by addition of carbon dioxide, and levels of strain KC were monitored for an additional 7 days.

CT Transformation in Schoolcraft Aquifer Solids

To evaluate the ability of strain KC to colonize and degrade CT in aquifer solids, nine columns were packed with aquifer material from the Schoolcraft aquifer. The columns were exchanged with Schoolcraft groundwater containing 20 to 30 μg/L CT until saturated. On day 72 (after saturation was complete), the exchange water was supplemented with 0.6 mM NaOH, 0.6 mM acetate, and 0.1 mM potassium phosphate (1:1000 dilution of pH 8.0 100 mM buffer stock). The final pH was 8.1 to 8.3. One set of columns was inoculated with 4.7 x10^{10} strain KC cells (cfu) in 1 mL of 100 mM potassium phosphate buffer, pH 8.0. A second set served as an uninoculated control. A final set was chemically inhibited by addition of thimersol (200 mg/L). Columns were exchanged at 2.5 mL/min, and 5-mL fractions were collected and assayed for CT. The cumulative mass of CT degraded was calculated as per Siegrist and McCarty (1987). A complete description of these experiments is provided by Mayotte et al. (1995).

Experiments with Aquifer Materials from a CT-Contaminated Aquifer at Hanford, Washington

Synthetic groundwater and aquifer materials from a CT-contaminated aquifer at Hanford, Washington, was evaluated by dispensing 5-g samples of Hanford aquifer solids into 28-mL Balch tubes and adding 5 mL SGW medium at pH 6.7 or 8.2. The tubes were then supplemented with 730 mg/L acetate and 800 mg/L nitrate, flushed with argon, and sealed with Teflon-lined septa. CT was added as a sterile aqueous stock to a final concentration of 18 ppb. Samples received a 2% (v/v) inoculum of strain KC grown aerobically in medium D for 24 hours. CT was assayed as described previously. After 140 hours, samples were heated to 70°C for two hours to desorb sorbed CT, and the headspace was assayed for final CT levels.

RESULTS AND DISCUSSION

Kinetic Characterization of Strain KC and Indigenous Organisms in Schoolcraft Groundwater

Both strain KC and the Schoolcraft groundwater flora exhibited similar biphasic growth patterns at an initial pH of 8.2. In each case, nitrate was first converted to nitrite, and nitrite was subsequently converted, presumably to NO, N_2O, and N_2, although these gaseous products were not quantified. Kinetic parameters for each growth phase are summarized in Table 1. A critical difference between strain KC and Schoolcraft flora was in the observed yield. For the first phase of growth (nitrate to nitrite), the observed yield for strain KC was four times the observed yield for the Schoolcraft flora. This translated into a fourfold higher maximum specific growth rate for strain KC. A likely explanation for this yield difference is the competitiveness of strain KC under iron-limiting conditions. Dybas et al. (1995) have shown that strain KC produces a wide spectrum of iron-binding activities at pH 8.2. Separate experiments were conducted to estimate the half saturation coefficients K_s for nitrate utilization, assuming Monod kinetics (data not shown). These experiments gave a K_s value of 9.4$\pm$0.3 mg/L for Schoolcraft flora and 12.0$\pm$1.3 for strain KC, but the fit to the Monod model was poor.

For a single growth limiting substrate, competition theory predicts that the superior competitor will be the organism with the lowest resource requirement, as measured by the parameter R* (Tilman 1981). This parameter is identical to the parameter J of Hansen et al. (1980) and S_{min} of Rittman and McCarty (1980). It is the minimum resource (substrate) level where growth just balances decay, and is given by $S_{min} = K_s\,(b/(\mu_m - b))$, where b is the decay coefficient (d^{-1}). When K_s and b are similar for two competing populations, then the critical factor affecting the competition is μ_m. For KC, a low value would be expected for S_{min} because of its high specific growth rate at moderately alkaline pH levels.

Persistence of Strain KC in Schoolcraft Groundwater

Figure 1 illustrates the pH dependence of strain KC survival under no-growth conditions (no electron acceptor or donor). A stable population of stationary phase strain KC (10^3 to 10^5 cfu/mL) rapidly decayed upon a shift of pH from 8.2 to 7.5. The lethality of this pH drop may be due to changes in the speciation of trace copper, which was previously found to inhibit growth of strain KC growth at neutral pH levels (Criddle et al. 1990). It is notable that for replicate 1, some strain KC survived the pH shift, indicating that low levels may persist. Lewis and Crawford (1993) observed survival of strain KC in aquifer materials stored at 4°C for one year.

Aquifer Materials from a CT-Contaminated Aquifer at Schoolcraft, Michigan

Significantly more CT was removed in columns inoculated with strain KC than in uninoculated and killed controls (Table 2). Protein was measured in the effluent of the inoculated column, but protein was not detected in the effluent of the uninoculated control (data not shown). Thus, under moderately alkaline conditions, strain KC was able to colonize the Schoolcraft solids and transform CT.

Experiments with Aquifer Materials from a CT-Contaminated Aquifer at Hanford, Washington

As shown in Table 3, alkaline adjustment of the Hanford aquifer material slurries, followed by inoculation with strain KC resulted in CT degradation. Inoculated samples that received the alkalinity addition degraded three times more CT than inoculated samples without alkalinity addition.

Public Acceptance of Bioaugmentation

This paper illustrates that a temporary niche can be created to enable degradation of hazardous substances by introduced organisms. Some will argue that such strategies are ineffectual in gaining public acceptance for bioaugmentation. However, partially on the basis of evidence presented in this paper, State of Michigan regulatory officials are supporting use of *Pseudomonas* sp. strain KC in a field-scale bioaugmentation experiment at Schoolcraft, Michigan. The field experiment will address other important issues that bear on the practicality of bioaugmentation, including chemical and organism delivery to subsurface environments.

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REFERENCES

Criddle, C. S., J. T. DeWitt, D. Grbrić)-Galić), and P. L. McCarty. 1990. "Transformation of carbon tetrachloride by *Pseudomonas* sp. strain KC under denitrifying conditions". Appl. Environ. Microbiol. 56:3240-3246.

Dybas, M. J., G. M. Tatara, and C. S. Criddle. 1995. "Localization and characterization of the carbon tetrachloride transformation activity of *Pseudomonas* sp. strain KC". Appl. Environ. Microbiol. 61:758-762.

Hansen, S. R. and S. P. Huppel. 1980. "Single-nutrient microbial competition: qualitative agreement between experimental and theoretically forecast outcomes". Science 207, 1491-1493.

Lewis, T. A., and R. L. Crawford. 1993. "Physiological factors affecting carbon tetrachloride dehalogenation by the denitrifying bacterium *Pseudomonas* sp. strain KC". Appl. Environ. Microbiol. 59:1635-1641.

Mayotte, T., M. Dybas, and C. S. Criddle, 1995. "Bench-scale evaluation of bioaugmentation to remediate carbon tetrachloride-contaminated aquifer materials". Accepted for publication in Ground Water.

Rittman, B. E. and P. L. McCarty. 1980. "Model of steady state biofilm kinetics". Biotechnology and Bioengineering 22, 2343-2357.

Siegrist, H. and P. L. McCarty. 1987. "Column methodologies for determining sorption and biotransformation potential for chlorinated aliphatic compounds in aquifers". J. Contaminant Hydrology 2:31-50.

Stumm, W. and J. J. Morgan. Aquatic Chemistry, 2nd ed.; John Wiley & Sons: New York, 1981; p. 248.

Tatara, G. M., M. J. Dybas, and C. S. Criddle. 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, G. M., M. J. Dybas, and C. S. Criddle. 1995. "Rapid carbon tetrachloride transformation by diverse cell types in the presence of partially purified culture filtrate from *Pseudomonas* sp. strain KC". Batelle In-Situ and On-Site Bioreclamation. Third International Symposium, April 24-27, 1995. San Diego, California.

Tilman, D. 1981. "Transport-limited growth in the chemostat and its competitive inhibition; a theoretical treatment". Archiv. fur Mikobiologie 58: 145-154.

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TABLE 3. Transformation of CT in Hanford aquifer material soil slurries.

FIGURE 1. Persistence of strain KC under no-growth conditions in Schoolcraft groundwater at pH 8.2. After 2 weeks of incubation, the pH was reduced to 7.5.

KEYWORDS LIST

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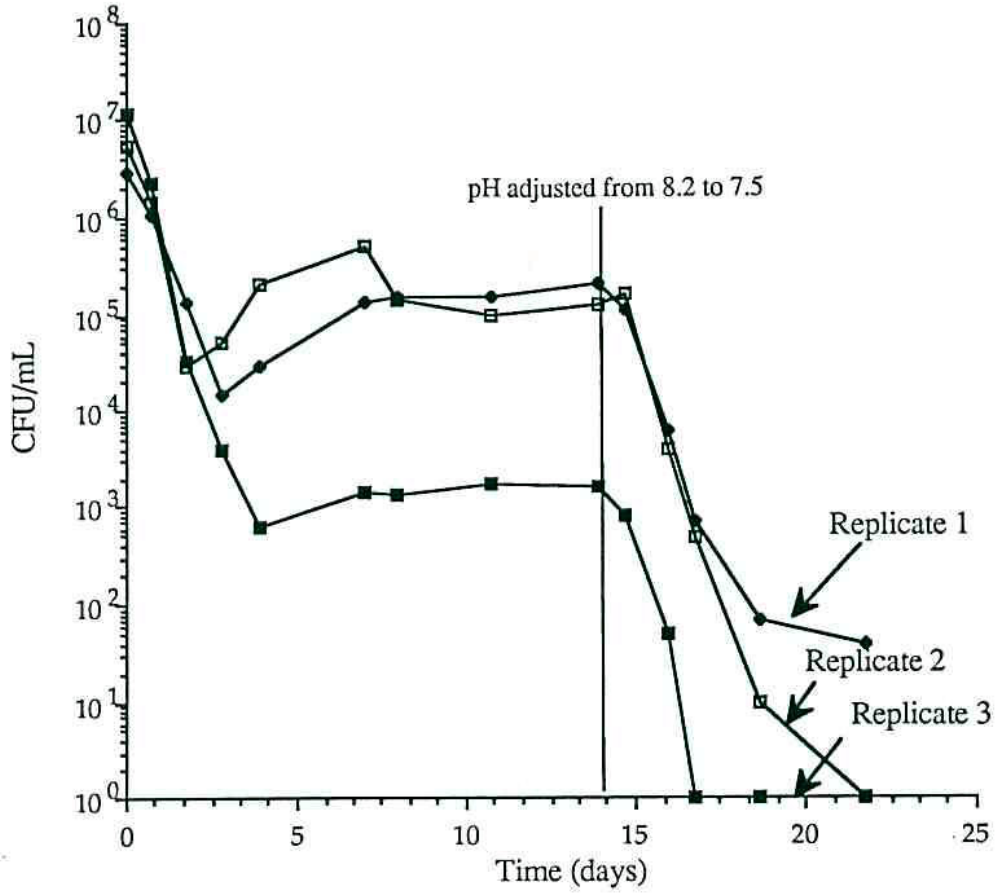

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TABLE 1. Kinetic parameters and yield coefficients for strain KC and Schoolcraft aquifer flora in Schoolcraft groundwater adjusted to an initial pH of 8.2.

Kinetic Parameter	KC[1]	Schoolcraft Flora[a]
Nitrate to nitrite		
μm, max. specific growth rate during NO_3^- conversion to NO_2^- (d^{-1})	3.12 ± 0.69	0.81 ± 0.21
Observed yield (mg cell dry weight per mg NO_3^- converted to NO_2^-)	0.21 ± 0.04	0.05 ± 0.01
Maximum specific rate of nitrate removal (mg NO_3^- per mg cell dry weight per day)	12.1 ± 1.8	11.7 ± 0.9
Nitrite to gaseous end products		
μm, max. specific growth rate during NO_2^- conversion to gaseous end products (d^{-1})	0.23 ± 0.09	0.67 ± 0.08
Observed yield (mg cell dry weight per mg NO_2^- reduced)	0.46 ± 0.18	0.18 ± 0.07
Maximum specific rate of nitrate removal (mg NO_2^- per mg cell dry weight per day)	0.59 ± 0.26	3.07 ± 0.50
Nitrate to gaseous end products		
Overall observed yield (mg cell dry weight per mg NO_3^-)	0.40 ± 0.04	0.12 ± 0.03

[a] Average ± one standard deviation for three independently grown cultures at 21.1°C.

TABLE 2. Removal of CT in batch columns containing Schoolcraft aquifer material exchanged with niche-adjusted groundwater (pH 8.1 - 8.3).

Column Description	Cumulative CT Removed Between Exchange Events (μg)[a]
Inoculated with strain KC	4.00 $\pm$ 0.50
Uninoculated control	1.90 $\pm$ 0.50
Thimersol inhibited control	0.80 $\pm$ 0.30

[a] Average $\pm$ one standard deviation for triplicate columns. Columns were incubated for 120 days.

TABLE 3. Transformation of CT in Hanford aquifer material soil slurries.

	CT Removed (μg)[a]	
Organism(s)	**pH 6.7[b]**	**Ph 8.2[c]**
Pseudomonas sp. strain KC	0.043 $\pm$ 0.053	0.185 $\pm$ 0.039
Uninoculated control	0.054 $\pm$ 0.011	0.024 $\pm$ 0.073

[a] Determined after 140 hr incubation at 20°C.
[b] Average $\pm$ one standard deviation for 3 independent samples.
[c] Average $\pm$ one standard deviation for 4 independent samples.