# Tracking the Activity of Bacteria Underground

***Livermore researchers use molecular biology and analytical chemistry to study the progress of bacteria in cleaning up pollutants in aquifers.***

WITH the recent focus on biological warfare agents that can cause diseases such as anthrax or plague, the myriad ways that bacteria can benefit people are easily overlooked. In the area of cleaning up pollution, researchers are discovering the extraordinary capabilities of some bacteria to grow on and degrade substances, such as chlorinated solvents and benzene, that are quite toxic to humans. Many of these bacteria have been found in contaminated aquifers, leading to the discovery that naturally occurring bacteria can actually promote groundwater cleanup.

At Lawrence Livermore, environmental microbiologists Harry Beller and Staci Kane are interested in the natural role of bacteria in breaking down compounds of environmental concern. They head up an applied research team that is part of the Environmental Protection Department's efforts to understand how these unusual bacteria can naturally degrade the compounds that escape from leaking underground fuel tanks (LUFTs). To get a better handle on how these bacteria work in the subsurface, researchers are using two advanced techniques for rapidly and reliably detecting the bacterial degradation of toxic compounds in soil and groundwater samples collected at LUFT sites.

One technique, real-time polymerase chain reaction (PCR), is widely used in biomedical research and has also been applied at Livermore to detect bioterrorism agents. The other technique, liquid chromatography/tandem mass spectrometry (LC/MS/MS), is also used to detect chemical warfare agents and in the biomedical and pharmaceutical industries. Both techniques show great promise for monitoring the activity of bacteria that are cleaning up groundwater naturally.

## What Lurks Below the Surface

Groundwater contamination at LUFT sites is a pervasive problem at federal and commercial facilities throughout the U.S. By 2002, more than 427,000 releases from LUFTs had been confirmed nationwide, and according to the U.S. Environmental Protection Agency, the cleanup backlog totaled more than 142,000 sites. Among the compounds in gasoline that are of greatest regulatory concern are benzene, toluene, ethylbenzene, and the three xylene isomers. (Isomers are compounds that have the same atomic composition but differ in structural arrangement.) BTEX is the acronym for this mixture of hydrocarbons. BTEX are among the most toxic and water-soluble constituents of gasoline.

Bioremediation is one of several accepted methods for restoring BTEX-contaminated aquifers to environmentally satisfactory conditions. Intrinsic bioremediation (also called natural attenuation) relies on indigenous bacteria to degrade contaminants in place and is a cost-effective approach favored by the LUFT owners responsible for cleanup. However, regulatory agencies and the public are sometimes skeptical of intrinsic bioremediation, viewing it as a "do nothing" approach.

The key to acceptance of intrinsic bioremediation at a given LUFT site is the ability to demonstrate in a substantial, scientifically credible manner that biodegradation of the contaminants is occurring. As Beller notes, "One of the roadblocks to the widespread acceptance of intrinsic bioremediation in groundwater is the difficulty of proving that measured decreases in BTEX concentrations are due to bacterial degradation and not to nondestructive processes such as dilution or dispersion. The monitoring methods that we have developed are designed to be capable of providing incontrovertible evidence of the biodegradation of BTEX compounds."

## How Bacteria Remediate Naturally

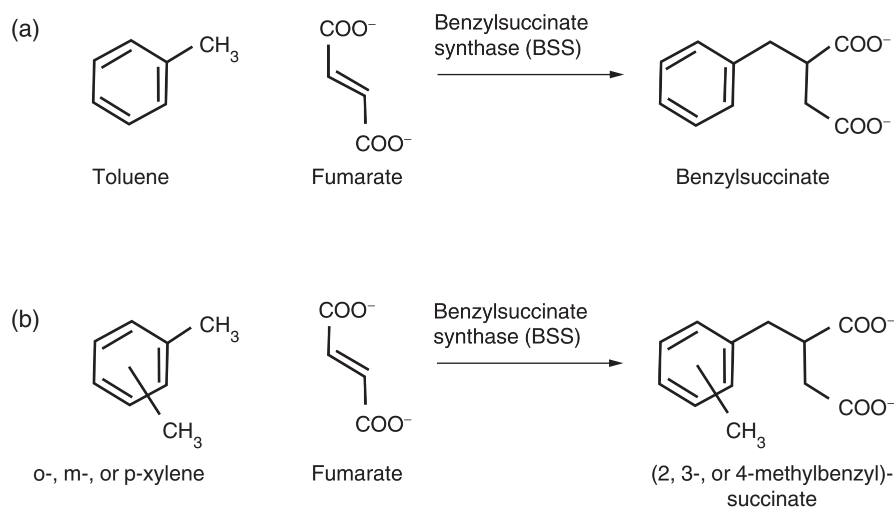
Groundwater underlying LUFT sites is typically oxygen-depleted (anaerobic) because oxygen-respiring (aerobic) bacteria rapidly use up the available oxygen. Once the oxygen in the groundwater environment is depleted, intrinsic bioremediation can only work if anaerobic bacteria are present that can degrade BTEX. As recently as 15 years ago, notes Beller, conventional wisdom held that anaerobic BTEX-degrading bacteria didn't exist because degradation of BTEX compounds in the absence of oxygen was an insurmountable biochemical challenge.

Groundbreaking research in the past seven years not only showed that such bacteria exist, but it also identified the key enzyme, benzylsuccinate synthase (BSS) that carries out the first step of anaerobic degradation of toluene and xylenes. During this time, researchers discovered the gene sequences that lead to the production of this enzyme. The BSS reaction (see the figure below) is found in diverse anaerobic, toluene-degrading bacterial cultures that represent the range of bacteria one would expect to find in aquifers under LUFT sites.

Beller and Kane decided to leverage this new understanding of anaerobic BTEX degradation to develop methods that would unequivocally demonstrate whether intrinsic BTEX bioremediation was occurring at a given LUFT site. Their methods focus on two aspects of the metabolic process.

The first monitoring method focuses on the *bssA* gene. This method gives scientists a tool for counting the bacteria that harbor a gene specific to anaerobic toluene and xylene degradation. The second method focuses on the unique metabolic products of the BSS reaction, which are benzylsuccinate and methylbenzylsuccinates. This method sensitively detects these metabolites, which have no known sources other than the anaerobic degradation of toluene or xylenes.

Beller explains, "The power of these so-called signature metabolites is that their mere presence in groundwater definitively demonstrates the degradation of specific compounds. There is no other way that these compounds could appear in the groundwater."




The benzylsuccinate synthase (BSS) reaction, which anaerobic bacteria use to attack (a) toluene and (b) xylenes as the first step of the biodegradation process. The BSS enzyme catalyzes the addition of toluene or xylene to fumarate, a compound that is typically present in many bacteria.

## Monitoring Bacteria Genetically

The first method quickly and accurately counts the number of copies of a specific bacterial gene, *bssA*, in samples of aquifer sediment. Because each bacterial cell typically contains only one copy of the *bssA* gene, the number of gene copies is equivalent to the population of bacteria that contain *bssA*. Thus, this method quantifies the number of bacteria that are genetically capable of anaerobic toluene or xylene degradation in a given sample.

"Because the BSS pathway is the only one to date that has been identified with anaerobic toluene degradation, measuring populations of bacteria containing *bssA* probably is inclusive of most anaerobic toluene- and xylene-degrading bacteria," says Kane.

How does counting the copies of *bssA* relate to intrinsic bioremediation? Kane explains that if anaerobic bacteria at a LUFT site are metabolizing BTEX and proliferating, it is reasonable to expect that the populations of these bacteria should be higher within BTEX-contaminated areas than in nearby, uncontaminated areas. "This method allows us to compare bacterial populations containing *bssA* over distance or time," she adds.

The researchers use analysis based on the real-time PCR—also known as quantitative PCR or TaqMan® PCR—to quantify copies of the *bssA* gene by targeting DNA sequences that are unique to this gene. The same technology has been used extensively by scientists at Livermore to develop real-time PCR methods for targeting specific bioterrorist agents such as *Bacillus anthracis*, which causes anthrax. (See the box, p. 18.)

The team, including microbiologist Tina Legler, began by comparing the *bssA* genes in four different toluene-degrading bacterial strains. At the time this study started, *bssA* sequences were only available for two strains, so *bssA* sequences were determined for two additional strains to provide a better assessment of the diversity of *bssA* sequences among toluene-degrading bacteria. In this and later studies, the team found a high degree of similarity

Region of predicted BssA sequence that includes a conserved glycine* residue

| Strain | Start | Sequence | End |
|---|---|---|---|
| GM GS-15*bssA* | | N I D H V Q F N C V S T A E M K A A Q K E P E K H Q D | |
| TA_K172*bssA* | 792 | N I D H V Q F N V V S T E E M K A A Q R E P E K H Q D | 818 |
| TA T1*tutD* | 795 | N I D H V Q F N V V S T D E M R A A Q R E P E K H H D | 821 |
| Asp_T*bssA* | 795 | N I D H V Q F N V V S T D E M R A A Q R E P E K H S D | 821 |

| Strain | Start | Sequence (* = G, 7th residue) |
|---|---|---|
| GM_GS-15*bssA* | | L I V R V S G F S A R F V D I P T Y G Q N T I I A R N E Q A F |
| TA_K172*bssA* | 819 | L I V R V S G F S A R F V D I P T Y G Q N T I I A R N E Q N F |
| TA_T1*tutD* | 822 | L I V R V S G Y S A R F V D I P T Y G Q N T I I A R Q E Q D F |
| Asp T*bssA* | 822 | L I V R V S G Y S A R F V D L P T Y G Q N T I I A R Q E Q D F |

Region of predicted BssA sequence that includes a conserved cysteine* residue

| Strain | Start | Sequence | End |
|---|---|---|---|
| GM_GS-15*bssA* | | P S I K H D V I G T E Q L K Y Y S Q F S K N N N G A T D D | |
| TA K172*bssA* | 451 | P S I K H N E L G V Q Q M L E M A K Y S R N G N G A T P E | 479 |
| TA_T1*tutD* | 454 | P S I K H D E I G T E Q M K E Y A K F S L N G N G A T D E | 482 |
| Asp T*bssA* | 454 | P S I K H D E I G T A Q M K E Y A K F S L N G N G A T D E | 482 |

| Strain | Start | Sequence (* = C, 10th residue) |
|---|---|---|
| GM_GS-15*bssA* | | E A H Y W G L V L C M S P G V C G R R K T H K T R S E G G G S I F |
| TA_K172*bssA* | 480 | E A H Y W V N V L C M A P G L A G R R K A Q K T R S E G G S A I F |
| TA T1*tutD* | 483 | E A H N W V N V L C M S P G I H G R R K T Q K T R S E G G G S I F |
| Asp T*bssA* | 483 | E A H N W V N V L C M S P G L H G R R K T Q K T R S E G G S S V F |

GM_GS-15*bssA* — *Geobacter metallireducens* strain GS-15, *bssA*
TA_K172*bssA* — *Thauera aromatica* K172, *bssA*
TA_T1*tutD* — *Thauera aromatica* T1, *tutD*
Asp_T*bssA* — *Azoarcus* sp. strain T, *bssA*

Evidence of the similarity of *bssA* gene sequences in four different bacterial strains. This figure shows the genetic code as "translated" into amino acids, which make up the enzyme. Only selected portions of the benzylsuccinate synthase enzyme are shown, specifically, portions of the largest of three subunits that make up the enzyme. Among the different bacterial strains, yellow indicates identical amino acids, and green indicates similar amino acids.

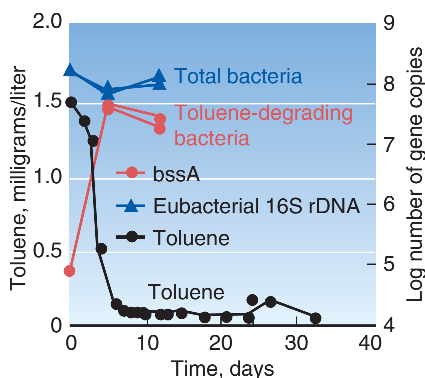
in this gene sequence among different organisms. (See the figure on p.17.) After aligning four DNA sequences chosen from their studies, the researchers focused on a stretch of DNA—about 130 base pairs—for development of their real-time PCR method. To target this region, they designed degenerate primers that corresponded to sequences from all four strains and an internal probe that complemented *bssA* from all four strains.

To test the technique, the team took samples from four sites with different histories of BTEX exposure, including three LUFT sites and an uncontaminated site. They spiked the sediments with BTEX, incubated them in the laboratory under various conditions, and monitored the BTEX degradation activity.

For real-time PCR analysis, they extracted and purified the total DNA from more than 100 5-gram sediment samples of these laboratory incubations. Using real-time PCR, the researchers successfully tracked bacterial population trends that were consistent with observed anaerobic toluene degradation activity.

They also discovered that, of all the environments studied, the ones with denitrifying conditions—that is, where nitrate was being respired by bacteria in the degradation process—had the most rapid toluene degradation and the largest abundance of *bssA*. In the samples with the most rapid toluene degradation, the numbers of *bssA* copies increased 100- to 1,000-fold during the first 4 days of incubation, the time when most of the toluene was being consumed. (See figure at left.) The team validated its method by comparing its results with those produced by traditional hybridization-based methods that do not use PCR amplification and by analyzing the sequences of PCR products to confirm the method's specificity.

The real-time PCR technique, Kane notes, has many advantages over other bacteria-counting methods that require cultivating the bacteria in the laboratory and then calculating the original populations. "Cultivating anaerobic bacteria can be a difficult, sometimes seemingly impossible, task. Because they reproduce slowly, cultivation is also time-consuming," she says. "Getting results can take from days to months, whereas the real-time PCR method does the job in less than an hour."




Real-time PCR of *bssA* successfully tracked toluene-degrading bacteria under denitrifying conditions. The relationship between toluene degradation and increases in numbers of *bssA* copies is apparent.

## Basics of the Real-Time Polymerase Chain Reaction

Developing a way to rapidly identify DNA by the real-time polymerase chain reaction (PCR) was a breakthrough event in the mid-1990s that launched Livermore's biodefense program. At the time, PCR was a well-established technique for identifying specific regions of DNA. PCR works by making multiple copies of a particular segment (referred to as the amplicon) of the DNA in the sample. When the sample is heated, the double-helix of DNA separates into two single complementary strands. When the sample is cooled, single, short (18- to 25-nucleotide) strands of DNA called primers attach to the ends of the target region to be amplified. Subsequently, a heat-stable enzyme (*Taq* DNA polymerase from *Thermus aquaticus*, a bacterium isolated from hydrothermal vents) replicates the region of DNA bracketed by the primers. With each heating–cooling cycle, the amount of DNA doubles. Eventually, after 20 cycles, a single target would be amplified a millionfold.

A dramatic advance in PCR technology was the development of real-time PCR, which allows for rapid quantification of specific genes. In addition to the specific primers used in conventional PCR, real-time PCR also includes a probe (typically 20 to 35 nucleotides long) that specifically binds to a region of the target DNA that is bracketed by the primers. The probe is labeled with fluorescent dyes at each end. One dye quenches the fluorescence of the other when the probe is intact. The real-time PCR method relies on the exonuclease activity of *Taq* DNA polymerase that cleaves the probe, resulting in fluorescence. The amount of fluorescence is proportional to the amount of replication, which in turn is proportional to the number of initial target DNA copies. By performing real-time PCR with specific DNA standards, a calibration curve is obtained to calculate the amount of target DNA in the environmental DNA extract.

The real-time PCR technique is fundamental to the Livermore-developed Handheld Advanced Nucleic Acid Analyzer (HANAA) and Biological Aerosol Sentry and Information System (BASIS), which are used to identify microorganisms that present a biological threat.

For more information about PCR and its biodefense applications, see *S&TR*, January/February 2002, pp. 24–26, and June 1998, pp. 4–9.

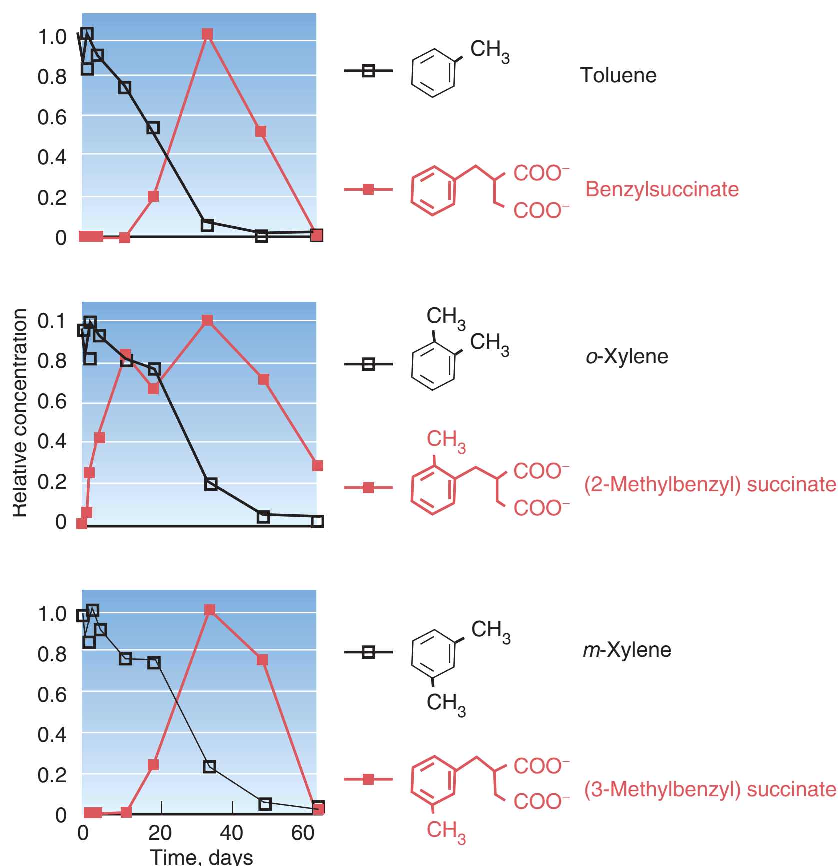
Another plus is the method's sensitivity. PCR can detect as few as five copies of a gene per analysis. It is also highly selective—an important quality, since it must avoid false-positive results.

## Detecting Key Signatures

The second method for tracking intrinsic bioremediation of BTEX in groundwater was developed by Beller, using LC/MS/MS to detect signature metabolites of BTEX degradation.

Instead of counting the populations of bacteria harboring a gene associated with BTEX degradation, this method measures distinctive metabolites (benzylsuccinate and methybenzylsuccinate isomers) that are uniquely associated with anaerobic toluene and xylene degradation.

Beller successfully used the signature metabolite approach in a controlled-release field study before coming to work at Livermore. (See the figure at right.) However, he didn't have access to LC/MS/MS then, and the more traditional analytical methods he used were labor-intensive and time-consuming. Traditional methods for such analysis typically involve extraction of a large (1-liter) water sample with organic solvent, concentration of the solvent to a small volume, chemical treatment called derivatization that makes the benzylsuccinates more amenable to further analysis, and gas chromatography/mass spectrometry analysis.




Concentrations over time of selected BTEX hydrocarbons and their corresponding benzylsuccinate metabolites in groundwater from a site in Seal Beach, California. In this experiment, BTEX and bromide were added to the groundwater at the sampling site. All concentrations are normalized to bromide (an inert tracer that corrects for the effects of dilution) and to the maximum concentrations of the compounds themselves. (Copyright 1995 by the American Chemical Society. Reused with permission from *Environmental Science and Technology*, 1995 **29**, 2869.)

Using the isotope-dilution LC/MS/MS method, Beller can skip the extraction, concentration, and derivatization steps and analyze a groundwater sample in less than 10 minutes. The method is highly sensitive, accurate, and precise, and it requires modest samples—less than 1 milliliter of groundwater. The detection limits for the LC/MS/MS technique are about 0.3 microgram of benzylsuccinate or methylbenzylsuccinate per liter (roughly the equivalent of a thimbleful of water in an Olympic-size swimming pool).

To test this method, Beller turned to a fuel terminal with contaminated groundwater. Since 1911, the terminal has been in the business of blending and distributing petroleum products, such as gasoline and diesel fuel. The team collected groundwater samples quarterly for a year from 12 wells located in the highly anaerobic aquifer. Methylbenzylsuccinates were detected in the three wells with the highest BTEX concentrations. The methylbenzylsuccinate concentrations ranged from less than 0.3 to 205 micrograms per liter. Beller found a strong and consistent correspondence between concentrations of methylbenzylsuccinates and their parent compounds, xylenes, throughout the most contaminated portion of the aquifer. (See figure on p. 20.)

Overall, the LC/MS/MS method proved to be a rapid, selective, and sensitive method for detecting benzylsuccinates, which are prime indicators of anaerobic bacteria hard at work degrading hydrocarbons.

## Future of "Natural" Bioremediation

A major challenge for the regulatory acceptance of intrinsic bioremediation is to provide evidence that decreases in the concentrations of groundwater contaminants truly represent biological metabolism of these contaminants rather than nondestructive, natural processes such as dilution. Beller and Kane have developed two independent methods to meet that challenge. They applied these techniques to contaminants at LUFT sites and are extending their use to other classes of contaminants, such as nitrate and high explosives.

Despite the significant strides that researchers have made in developing new methods for monitoring intrinsic bioremediation, a remaining challenge is to progress from qualitative evidence (is biodegradation occurring?) to quantitative evidence (what is the rate of in situ biodegradation, and what proportion of contaminant decreases can be attributed to biodegradation?). Beller and Kane are investigating ways to adapt their methods to yield more quantitative data.

An additional long-term goal of this research is to gain a better understanding of how ethanol—which is a strong contender to replace MTBE in gasoline—could affect the population of anaerobic BTEX-degrading bacteria and, therefore, the rates of intrinsic BTEX biodegradation in the subsurface. Before this project, notes Kane, researchers had no way to quickly assess the populations of anaerobic BTEX-degrading bacteria in a given environment. The real-time PCR technique, with its ability to quantify the abundance of the *bssA* gene, provides a tool for doing just that.

*—Ann Parker*

**Key Words:** benzene, toluene, ethylbenzene, xylene (BTEX); biodegradation; groundwater; hydrocarbons; intrinsic bioremediation; leaking underground fuel tank (LUFT); liquid chromatography/tandem mass spectrometry (LC/MS/MS); natural attenuation; polymerase chain reaction (PCR).

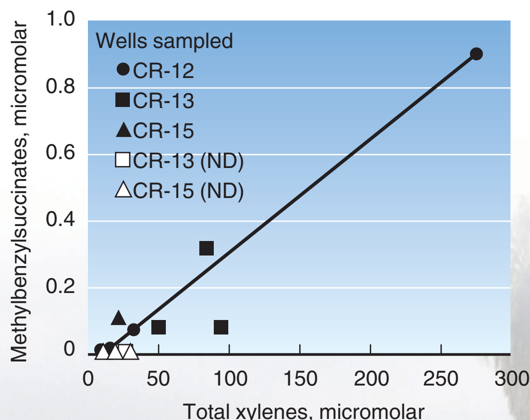
***For further information contact Harry Beller (925) 422-0081 (beller2@llnl.gov) or Staci Kane (925) 422-7897 (kane11@llnl.gov).***

**Additional Reading**

Harry R. Beller, Staci R. Kane, Tina C. Legler, and Pedro J. J. Alvarez (2002), "A Real-Time Polymerase Chain Reaction Method for Monitoring Anaerobic, Hydrocarbon-Degrading Bacteria Based on a Catabolic Gene," *Environmental Science and Technology* **26**, 3977–3984.

Harry R. Beller (2002), "Analysis of Benzylsuccinates in Groundwater by Liquid Chromatography/Tandem Mass Spectrometry and Its Use for Monitoring In Situ BTEX Biodegradation," *Environmental Science and Technology* **36**, 2724–2728.

Staci R. Kane, Harry R. Beller, Tina C. Legler, and Robert T. Anderson (2002), "Biochemical and Genetic Evidence of Benzylsuccinate Synthase in Toluene-Degrading, Ferric Iron-Reducing *Geobacter metallireducens*," *Biodegradation* **13**, 149–154.




Highly correlated concentrations of methylbenzylsuccinates (signature metabolites of xylenes) versus xylenes in groundwater from three different wells at four sampling times at a contaminated, fuel-distribution terminal. (Copyright 2002 by the American Chemical Society. Reused with permission from *Environmental Science and Technology,* 2002 **36**, 2727.)