Effect of Selective Pressure and Genetically Engineered Microorganism (GEM) Densities on Mercury Resistance (*mer*) Operon Transfer in Elbe River and Estuarine Sediments

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Bacterial reduction of mercury ions to elemental mercury by the *mer* operon-encoded microbial resistance mechanism has recently been shown to be a promising approach in the bioremediation of mercury-contaminated wastewater. Mercury resistance is widespread among environmental bacteria and several isolates have proven to be adaptable catalyzers for mercury reduction in bioreactors. To accomplish high, stable, and predictable performance, however, the genetically engineered microorganism (GEM) *Pseudomonas putida* KT2442::mer73 has been constructed, which constitutively expresses the mercury resistance operon at a high level, is nonpathogenic, and does not contain plasmids. To assess the safety of this GEM in an open environmental application, gene transfer was investigated in stream and estuarine microcosms containing sediments from the Elbe River and Roskilde Fjord, Denmark. In *P. putida* KT2442::mer73, the *mer*TPAB genes have been stably integrated into the chromosome to reduce the chance of horizontal transfer. Consequently gene transfer to an isogenic recipient strain, *P. putida* KT2442::Tc, could not be detected, although parameters such as recipient cell density, cell shock, continuous addition of cells, or application of mercury selective pressure were adjusted with respect to increasing the probability of gene exchange. On the basis of these experiments, the strain *P. putida* KT2442::mer73 can be regarded as safe.

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Mercury (Hg) has been one of the most prominent contaminants of the Elbe River, which flows through Germany and the Czech Republic, and which used to be one of the most polluted rivers in the world. Until 1991, Elbe River mercury concentrations in suspended particulate matter (SPM) were above 25 mg/kg at the measuring station in Schnackenburg, 475 km downstream of the Czech border [Arbeitsgemeinschaft (ARGE) Elbe, 2004b]. Current online measurements, however, show mercury concentrations in SPM below 5 mg/kg and between 20 and 100 ng/L in Elbe water (Elsholz et al., 2001), which is more than tenfold below the permissible concentration for drinking water (1000 ng/L). Although the present mercury burden may seem low, the danger of mercury concentration via the food chain remains and poses a threat to humans regularly consuming fish from the Elbe River (Lommel, Kruse, and Wassermann, 1985).

Mercury contamination of the Elbe was mainly a result of industrial processes utilizing mercury (e.g., the chlor-alkali electrolysis process for the production of chlorine), as wastewater was disposed of without cleanup. An alternative to using expensive chemical mercury remediation (e.g., ion exchange columns) has been developed on the basis of microbial mercury reduction by Wagner-Döbler et al. (2000). Here, mercury-resistant bacteria grow as biofilms on porous carrier material in bioreactors that are continuously fed with mercury-contaminated wastewater supplemented with sucrose to allow bacterial growth. Ionic mercury is detoxified by the bacteria by conversion to nontoxic, insoluble elemental mercury (Hg^{o}), which diffuses out of the cell and is captured in the bioreactor carrier material. The genes necessary for this process are encoded on the so-called *mer* operon (mercury resistance operon).

Although a great percentage of natural bacteria possess mercury resistance systems (Osborn et al., 1997), genetically engineered microorganisms (GEMs) may be provided with advantageous characteristics, such as high and constitutive expression of the mercury resistance. Horn et al. (1994) have constructed several *Pseudomonas* strains

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with this trait (Canadian patent: CA 2104502), of which *Pseudomonas putida* KT2442::mer73 appeared to be the most promising candidate showing highest resistance to mercury (up to 80 ppm phenyl mercuric acetate added at the midlogarithmic phase). Moreover, when grown with mercury-containing chlor-alkali wastewater, the strain showed highest mercury transformation rates when compared with mercury-resistant wild-type isolates (von Canstein et al., 2002).

Since the first utilization of GEMs for industrial processes and in agriculture, there has been discussion and, to a great extent, concern and skepticism about the safety of the newly created strains in case of an accidental or deliberate release into the environment (e.g., Kellenberger, 1994; Sheridan, 2000; Stotzky and Babich, 1984). In Europe, these concerns have resulted in Directive 2001/18/EC of the European Parliament and of the Council of March 12, 2001, on the deliberate release of genetically modified organisms into the environment. Besides good knowledge and a description of the donor and recipient [or parental organism(s)] and the vector(s) used in the construction, the directive requires a documented description of the ecology and environmental impact of a genetically modified organism, and of the persistence of the recombinant DNA and its transfer to the indigenous microflora. *P. putida* KT2442::mer73 was constructed with regard to minimizing the chance of gene transfer: the *mer* operon was integrated into the chromosome via transposon mutagenesis, but without the integration of the transposase, thus creating genetically stable constructs. In addition, *P. putida* KT2442 does not contain plasmids; thus mobilization of the chromosomally integrated genes into a plasmid via transposition or recombination is highly unlikely. Nevertheless, experimental proof of a lack of gene transfer in *P. putida* KT2442::mer73 is crucial.

Mechanisms for the mobilization of genetic material are conjugation, transformation, and transduction, all of which have been shown to occur in the environment (Davison, 1999). Gene transfer frequencies are strongly dependent on the specific habitat and the parameters of the experimental setup, and they can be as high as 0.34 per recipient (see Ashelford et al., 1997, for a list of gene transfer results in simple microcosms). Besides high cell densities, conditions of selective pressure favor the dissemination of the introduced genes, both by influencing directly the probability of the gene transfer event, and by subsequent increased growth of the new phenotype (de Lipthay, Barkay, and Sørensen, 2001; Rasmussen and Sørensen, 1998).

A microbiological cleanup technology utilizing recombinant mercury-reducing bacteria would most likely release GEMs at a low density continuously into a river, and from there into the sea, which represents a completely different habitat. We present here the results of stream and estuarine microcosm experiments in which the GEM *P. putida* KT2442::mer73 was investigated with regard to *mer* operon gene transfer into a tetracycline-resistant isogenic recipient (*P. putida* KT2442::Tc) in two ecologically different habitats, that is, in stream (Elbe River) or estuarine (Roskilde Fjord) sediments. To increase the probability of gene transfer and to test different release scenarios, the effects of cell shocks and selective pressure were also investigated.

Materials and Methods

Stream Microcosm Experiments

Stream Microcosms

The design and operation of the microcosms has been described elsewhere (Pauling and Wagner-Döbler, 2003). The microcosms were filled with approximately 12.5 L of Elbe River water that was recycled for 12 days (so that the disturbed microbial community could reestablish). After that period, the water was continuously replaced with tap water at a refreshment rate of 15 ml/min (which translates into a complete exchange of water within 14 hours). The main chamber was filled to a depth of approximately 4 cm with Elbe River sediment. The microcosms were kept at room temperature (20° C–25° C); water pH was 7, as determined with pH indicator strips (Merck, Darmstadt, Germany; pH 0–14). To control primary production, the sidewalls of the troughs were darkened with paper cartons and a plant light employed to render a 12-hour light/12-hour dark cycle.

Elbe River Sampling

Water and sediment were collected in October 2000 from the Elbe River near Magdeburg, Germany, near a measuring station 318 km downstream of the Czech border. Water temperature was 11.6° C, pH 8.3, and oxygen content 10.7 mg/L. The acid-soluble mercury concentration in water was 0.06 ppb [Arbeitsgemeinschaft (ARGE) Elbe, 2004a]; the mercury concentration of SPM-borne sediment was approximately 7 mg/kg (July 2000). The water was added to the microcosm untreated. The sediment was filtered through a 5 mm sieve before addition to the microcosm.

Background levels of mercury- and antibiotic-resistant Elbe River bacteria (Table 1) were determined by spreading

Table 1. Natural background levels of mercury- and antibiotic-resistant bacteria in Roskilde Fjord and Elbe River water and sediment

Growth medium	Average water sample (SD) [CFU/ml]	Average sediment sample (SD) [CFU/g]
Estuarine water and sediment		
Luria Bertani	8.2×10^3 (3.9×10^3)	3.8×10^5 (4.3×10^5)
Luria Bertani-Hg^{2+}	2.0×10^2 (1.7×10^2)	1.8×10^3 (7.0×10^2)
Luria Bertani + phenyl mercuric acetate	1.3×10^1 (1.8×10^1)	3.0×10^2 (2.8×10^2)
Luria Bertani + tetracycline	bd	bd
Luria Bertani + rifampicin	bd	bd
Elbe River water and sediment		
R2A	9.0×10^3 (0)	7.3×10^5 (4.4×10^5)
R2A + phenyl mercuric acetate	bd	bd
R2A-tetra-cycline	bd	9.0×10^2 (5.5×10^2)
R2A-rifampicin	bd	bd

Samples were spread on agar plates directly after Roskilde Fjord or Elbe River sampling.
SD = standard deviation; duplicate samples are presented for estuarine microcosms and triplicate samples for stream microcosms.
bd = below detection.

triplicate samples on selective medium (R2A containing 10 ppm of mercury as phenyl mercuric acetate; 10 μg/ml tetracycline or 100 μg/ml rifampicin). See below for a detailed description of the enumeration of bacteria.

Experimental Design Including Cell and Mercury Shock

Of three parallel microcosms, one (MCI) was inoculated with *P. putida* KT2442::mer73 (donor) and *P. putida* KT2442::Tc (recipient). In addition, this microcosm was amended with 250 μg/L phenyl mercuric acetate. The second microcosm (MCII) was inoculated with the donor and the recipient, but mercury was not added. The third microcosm (MCIII) served as an untreated control. It remained uninoculated and was not provided with mercury. After the recycling period, bacteria were inoculated continuously into MCI and MCII. At day 48, a cell shock with high cell densities [10^8 colony forming units (CFU)/ml] was presented to MCI and MCII. At day 53, 10 ppm of mercury (as phenyl mercuric acetate) were added to MCI (mercury shock).

Inoculation of Stream Microcosms

The microcosms MCI and MCII were continuously inoculated from two chemostats of the donor *P. putida* KT2442::mer73 and the recipient *P. putida* KT2442::Tc, respectively. The chemostats, consisting of 1 L Schott bottles with an olive at the bottom of the flask (for withdrawal of culture suspension) and another olive above the 1 L mark (for attaching a sterile air filter) were supplied with M9 minimal medium (Wagner-Döbler et al., 1992) that was amended with 10 mM sodium benzoate at approximately 30 ml/hr. Medium was added via a sterile steel needle through a silicon seal matching the Schott polybutylene terephtalate (PBT) lids. A stirrer was used for good mixing and aeration of the culture. The sterile air filter allowed for sterile air to be respired. Cell densities in the two chemostats were approximately 10^7 CFU/ml for *P. putida* KT2442::mer73 and 10^8/ml for *P. putida* KT2442::Tc. Inoculation with bacteria was accomplished by inserting silicon tubing from the chemostat with a long needle (Braun, Melsungen, Germany, Sterican, 0.90 × 70 mm, 20 G × 2 4/5″ Luer Lock) into the microcosm through the middle of three 15 mm diameter rubber stoppered holes at the upstream head side.

Enumeration of Bacteria

Aliquots (100 μl) from three serial dilutions (in 1% NaCl) of fresh water or sediment samples from the microcosms were spread in triplicate on solid R2A (Fluka Chemie, Buchs, Switzerland) agar medium for the determination of total CFU. For the determination of mercury- or tetracycline-resistant bacterial densities, samples were spread on solid R2A agar containing 5 ppm Hg ($HgCl_2$) or 10 μg/ml tetracycline. Donor and recipient densities were determined by spreading three serial dilutions of the samples in triplicate on solid M9 medium containing 10 mM sodium benzoate (Fluka, Buchs, Switzerland) as the sole carbon source, 100 μg/ml rifampicin, and 1 ppm Hg ($HgCl_2$) or 1

μg/ml tetracycline, respectively; or, for the detection of transconjugants, the plate contained 100 μg/ml rifampicin, 1 ppm Hg as $HgCl_2$, and 10 μg/ml tetracycline.

Sediment samples were taken with a sterile 5 ml plastic pipette equipped with a blunt end. The pipette was inserted into the sediment at the center of the microcosm and approximately 5 ml of sediment were drawn in and transferred into a 50 ml tube (Greiner, Frickenhausen, Germany). It was weighed (wet), mixed with phosphate buffer (PBS, per 1 L: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, pH 7.3) in a 1:1 [w(g):v(ml)] ratio, vortexed for 1 minute at full speed, sonicated in a water bath (Bandelin Sonorex Super RK510H) for 2 minutes, and again vortexed for 1 minute at full speed. When the sediment had settled, 1.5 ml of the supernatant were transferred into a reaction tube and a dilution series prepared as described above. Spreading of water or sediment samples from the uninoculated microcosm did not yield colonies on selective plates except for sediment samples on M9-benzoate medium with tetracycline (see below). The detection limit was at 10 CFU/ml.

Estuarine Microcosm Experiments

Estuarine Microcosms

Gene transfer in estuarine sediment was investigated in microcosms that have been described previously (Kroer and Coffin, 1992; Kroer, Coffin, and Jørgensen, 1994). The experiments were performed at 20° C and 250 μEcm^2/min light intensity with a 12-hour light/12-hour dark cycle and the microcosms were calibrated for approximately 24 hours before inoculation. Experiments to elucidate the effect of selective pressure on gene transfer were conducted using a small-scale design of the 27 L microcosms described by Barkay et al. (1995) and Sørensen et al. (1996). The microcosms were filled with 9 ml of water and 0.1–0.2 g of sediment and were amended with 250 μg/L phenyl mercuric acetate.

Roskilde Fjord Sampling

Marine samples were taken with a glass corer (6 mm internal diameter) at a water depth of 20–25 cm from Roskilde Fjord, off the pier at Risø National Laboratory, Denmark. The upper 1 cm of the sediment was sampled; the water temperature was 8.5° C and salinity was 0.6% on the day of sampling for the first experiment. For the second and third experiment, temperature and salinity were 13° C/0.8% and 9° C/0.6%, respectively. For the determination of background levels of mercury- and antibiotic-resistant bacteria in Roskilde Fjord, duplicate samples both of water and sediment were taken at a depth of approximately 15 cm (Table 1). Of the sediment, 100 mg were extracted in 900 μl PBS (per 1 L: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4; pH adjusted to 7.4 with HCl) in Eppendorf reaction tubes (i.e., vortexed for 1 minute at full speed) and 200 μl spread on selective medium, both of water and sediment samples.

Gene Transfer After Release of a Large Number of Bacteria

Triplicate microcosms were inoculated with approximately 5×10^6 CFU/ml final density of the donor *P. putida* KT2442::mer73-gfp-11, a fluorescent variant of the donor used in the stream microcosm experiments, and 5×10^6 CFU/ml final density of the recipient *P. putida* KT2442::Tc. Before addition, all inoculants were washed three times with PBS (6,000 g, 10 minutes, 10° C). The microcosms were operated for 21 days and samples taken every other or third day from day 1 on. In this experiment, gene transfer not only to the recipient but also to indigenous phenol-degrading bacteria was investigated. Samples were spread on solid Luria Bertani medium (per 1 L: 10 g NaCl, 10 g tryptone, 5 g yeast extract, 15 g agar) containing 15 μg/ml tetracycline and 25 μM $HgCl_2$ (3.7 ppm Hg) or on minimal medium (per 1 L: 4.24 g $K_2HPO_4 \times 3H_2O$, 1 g NaH_2PO_4, 2 g NH_4Cl, 0.123 g disodium nitriloacetic acid, 0.2 g $MgSO_4$, 0.012 g $FeSO_4 \times 7H_2O$, 0.003 g $ZnSO_4 \times 7 H_2O$, 0.003 g $MnSO_4 \times H_2O$, pH 7) with 25 μM phenyl mercuric acetate (5 ppm Hg) to determine transconjugants, or with 25 μM phenyl mercuric acetate and phenol to determine mercury-resistant, phenol-degrading bacteria in the sediment. A few drops of phenol were transferred to a small test tube. The tube was suspended from the lid of the petri dish by use of tape. The petri dish was then sealed with parafilm and the phenol degraders grew on the vapors inside the dish.

Enumeration of donor and recipient was achieved by spreading appropriate dilutions of 200 μl on solid Luria Bertani medium containing 100 μg/ml rifampicin, or on Luria Bertani medium with 15 μg/ml tetracycline.

Gene Transfer to Recipient at Continuous Release of Bacteria

For this experiment the microcosms were inoculated daily to render densities of approximately 10^3 CFU/ml, both of donor and recipient, except for day 21, when microcosms

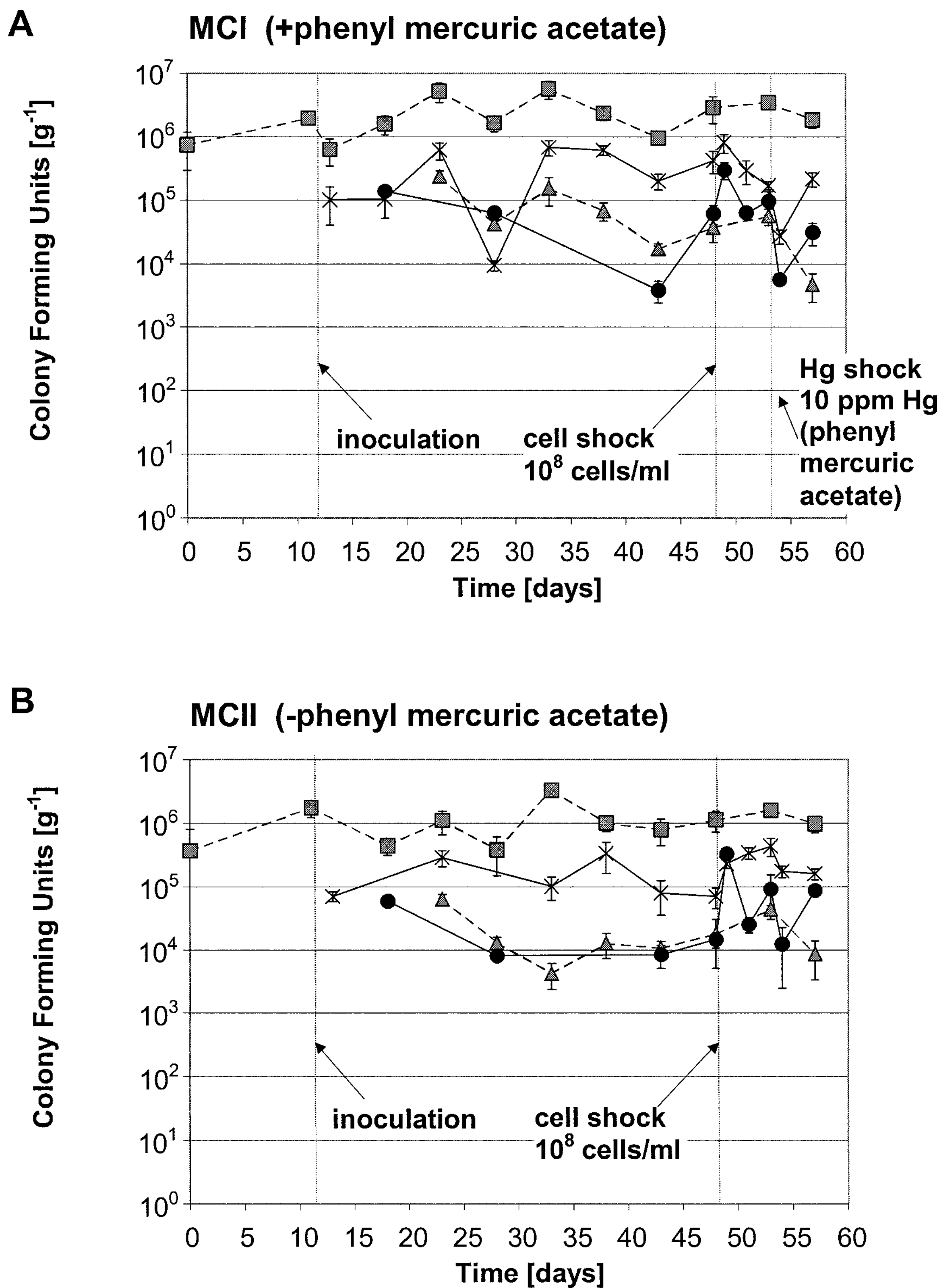

Figure 1. Gene transfer in stream microcosms. Bacterial densities in sediments are shown for stream microcosms inoculated with the donor *P. putida* KT2442::mer73 and recipient *P. putida* KT2442::Tc. The upper graph (A) shows the colony forming units (CFU) for the microcosm that was amended with phenyl mercuric acetate. The lower graph (B) shows the CFU in the unamended microcosm. Total bacterial densities are depicted with a square (– -■– -), mercury-resistant microorganisms with a triangle (- –▲– -), and donor and recipient are represented by a filled circle (-●-), and a cross (-✕-), respectively. Inoculation began after a recycling period

were not inoculated (termination of experiment). Only gene transfer to the recipient was investigated. Samples were taken and enumeration of donor, recipient, and transconjugants was carried out as described above.

Gene Transfer to Recipients During Continuous Release of Bacteria and Under Selective Pressure

For this experiment the microcosm was amended with 250 μg/L of phenyl mercuric acetate. Inoculation was performed daily with approximately 10^3 CFU/ml of both the donor and recipient. Samples were taken as described above and bacterial densities determined as in the other two experiments.

Controls

For the first two experiments (effect of cell density), a control microcosm was inoculated with the same final densities of the nonrecombinant parent strain *P. putida* KT2442 and the recipient *P. putida* KT2442::Tc. The control for the last experiment (selective pressure) was a microcosm inoculated with donor and recipient, but without selective pressure.

Results

Gene Transfer Experiments in Stream Microcosms

Gene transfer in a simulated river environment was investigated, including the influence of a cell shock and selective pressure (Figure 1). Three microcosms were run in parallel, of which one (MCI) was inoculated with both the donor (*P. putida* KT2442::mer73) and the recipient (*P. putida* KT2442::Tc). Phenyl mercuric acetate was added to the inflowing tap water to a final concentration of 250 μg/L. The second microcosm (MCII) was also inoculated with both donor and recipient, but did not contain any mercury. The third (MCIII) microcosm was operated without the addition of donor and recipient and without mercury. During the experiments, donor and recipient were introduced continuously from two chemostats to yield densities between 10^4 and 10^6 CFU/g sediment (wet weight). On day 48, a cell shock was performed in MCI and MCII with 10^8 donor cells/ml in the water column. On day 53, phenyl mercuric acetate was added to the first microcosm to give a concentration of 10 mg/L mercury.

←
on day 12; on day 48, a cell shock was performed, adding donor cells to give a density of 108 donor cells/ml; a mercury shock yielding 10 ppm of mercury (as phenyl mercuric acetate) was applied on day 53. Note that over the duration of the experiment, no transconjugants (resistant to mercury and tetracycline) could be detected.

Effect of Phenyl Mercuric Acetate on Bacterial Densities and Gene Transfer

Total bacterial numbers in MCI and MCII were approximately 10^6/g over the duration of the experiment (57 days) and slightly higher in MCI (but well below 10^7/g). The control microcosm, MCIII, also retained 10^6 CFU/g over the course of the experiment (data not shown; for natural background see Table 1). This is within the range previously found in uninoculated aquatic sediment microcosms with sediments from the Rhine River, Germany (Pipke et al., 1992).

Mercury-resistant bacteria, both in MCI and MCII, were mainly made up of the donor. In the control microcosm (MCIII), mercury-resistant bacteria yielded only between 10 and 100 CFU/g (data not shown; for natural background see Table 1) as determined on R2A agar with 5 ppm mercury (as phenyl mercuric acetate). For soil, much higher frequencies have been determined (Smit, Wolters, and van Elsas, 1998). Others, however, have found mercury-resistant cell densities in soil (Ranjard et al., 2000) or marine sediments (Rasmussen and Sørensen, 1998) within the same range.

Donor numbers in MCI were between 10^4 and 10^5 CFU/ml (+ phenyl mercuric acetate), that is, up to one order of a magnitude greater than in MCII (no mercury). A possible explanation for this is the competitive advantage the mercury-resistant donor strain may have compared with the natural population under selective pressure.

Recipient numbers were determined to be a magnitude higher compared with donor numbers in both MCI and MCII, despite identical treatment with regard to cultivation and inoculation to the microcosms. This was a consequence of different cell densities in the chemostats in favor of the recipient (10^8 CFU/ml versus 10^7 CFU/ml). Rochelle, Fry, and Day (1989) found highest frequencies for conjugal gene transfer of a mercury-encoding plasmid from a mixed natural suspension of epilithic bacteria to *Pseudomonas* sp. at donor:recipient ratios of 0.12×10^{-3} and 1.7×10^{-3}; therefore it may have been favorable, for gene transfer to occur, to have a surplus of recipients. In the control microcosm, recipient selective plates showed a background of between 100 and 1000 CFU/g (data not

shown; for natural background see Table 1). Recipient cell densities in the inoculated microcosms, however, were generally two to three orders of magnitude higher; thus the background did not affect the results.

Transconjugant colonies were not found for the duration of the experiment. On selective medium for false positives, transconjugant colonies could sometimes be observed, especially if too much sediment was plated out together with the supernatant. Those bacteria failed to grow, however, if transferred to fresh medium containing mercury and tetracycline.

Influence of the Cell Shock

Cell shock raised donor CFU densities temporarily but did not have any notable effect on gene transfer. No transconjugant colonies could be detected, even under conditions of temporary donor surplus. It must be stated, however, that recipient numbers were high throughout and that the surplus of the donor only lasted for a maximum of one day. Nevertheless, the donor/recipient ratio was altered from approximately 10^{-1} to nearly 1 until the end of the experiment. This had no effect on gene transfer, however, and did not result in transconjugant colonies.

Influence of Mercury Shock

The mercury shock was applied as a selective force, enhancing the necessity for the microorganisms to acquire mercury resistance genes in order to survive. One day afterward, the recipient and donor CFU had dropped by at least one order of a magnitude, probably due to die-off as a direct consequence of the mercury shock. Since the total exchange of water in the microcosm was accomplished within 14 hours, it is not surprising that cultivatable bacterial numbers recovered quickly. This included total CFU and mercury-resistant CFU that were not determined until four days after the mercury shock. Until the end of the experiment, no transconjugant colonies could be recovered.

Gene Transfer Experiments in Estuarine Microcosms

Three gene transfer experiments were conducted in estuarine microcosms to determine the influence of donor and recipient densities and selective pressure on gene transfer. In the first experiment (Figure 2), the microcosms were inoculated with a final cell density of 5×10^6 CFU/ml, both of donor and recipient, at the beginning of the experiment. In the second experiment (Figure 3), the continuous release of small numbers of bacteria was investigated with the daily addition of approximately 10^3 CFU/ml. In the third gene transfer experiment (Figure 4), 250 μg/L of phenyl mercuric acetate were added and microcosms were inoculated daily with 10^3 CFU/ml.

Gene Transfer at Cell Shock

Donor and recipient numbers declined from approximately 10^6 CFU/ml to 10^2–10^3 CFU/ml in the water, and about tenfold—from 10^6 CFU/g to 10^5 CFU/g—in the sediment, over the course of the experiment (Figure 2). This suggests that even if inoculated at large numbers, the GEM or the recipient do not survive well in the water; they could, however, stabilize in the sediment. During this time, transconjugants were not observed.

Gene Transfer During Continuous Release of Small Cell Densities

Over the duration of this experiment (57 days), donor and recipient densities remained stable at approximately 10^3 CFU/ml in water and between 10^4 CFU/g and 10^5 CFU/g in the sediment (Figure 3). This suggests that the inoculants did not survive well in the water. A reduction in CFU numbers, by 50%, can even be seen on day 21, when the microcosms were not inoculated. In the sediment, a tendency for inoculant CFU to increase can be observed. No transconjugants could be obtained under these conditions either.

Gene Transfer Under Selective Pressure

Under selective pressure, CFU densities for donor and recipient increased initially but remained between 10^4 CFU/ml and 10^5 CFU/ml in the water and at approximately 10^5 CFU/g in the sediment (Figure 4). In the water, it is surprising to find the recipient reach the same densities as the donor. The same was found in the sediment. By the time the recipient reached donor densities, the phenyl mercuric acetate may have been transformed by the donor. In sediment, an increase in donor CFU density can be observed toward the end of the experiment. No transconjugants were observed. The generally elevated densities in the phenyl mercuric acetate-amended microcosms were possibly due to a die-off of grazers (nanoflagellates and ciliates) and potential bacterial competitors.

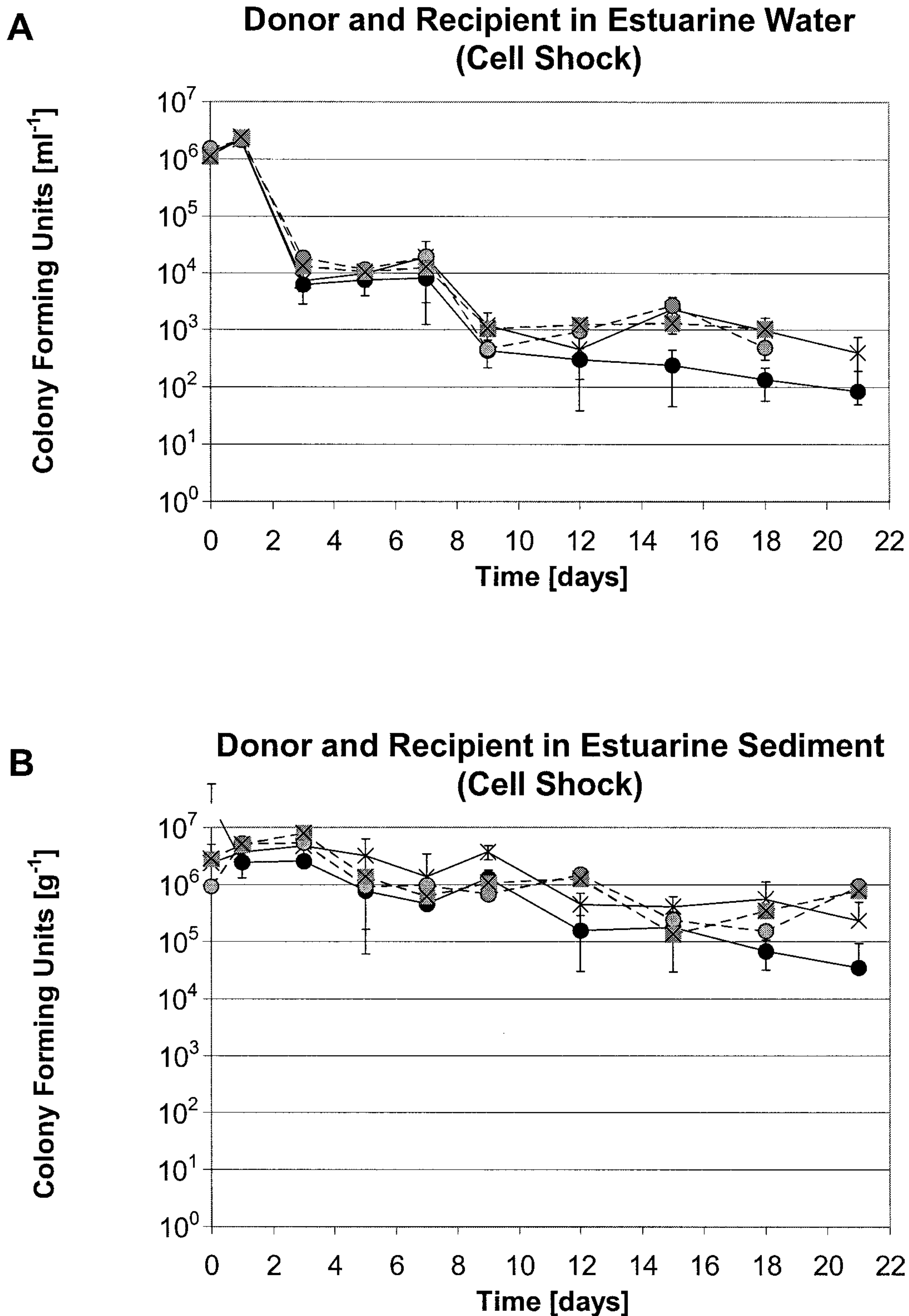

Figure 2. Gene transfer in estuarine microcosms after cell shock. Bacterial densities of the donor *P. putida* KT2442::mer73-gfp11 and the recipient *P. putida* KT2442::Tc in water and sediment of estuarine microcosms after cell shock (106 cells/ml) are shown. Donor and recipient are represented by a filled circle (-●-) and a cross (-✕-), respectively. Results from control microcosms are shown as well. These were inoculated with the parent strain *P. putida* KT2442 (– -◉- –) and the recipient (– -▩- –). No transconjugants were observed over the duration of the experiment.

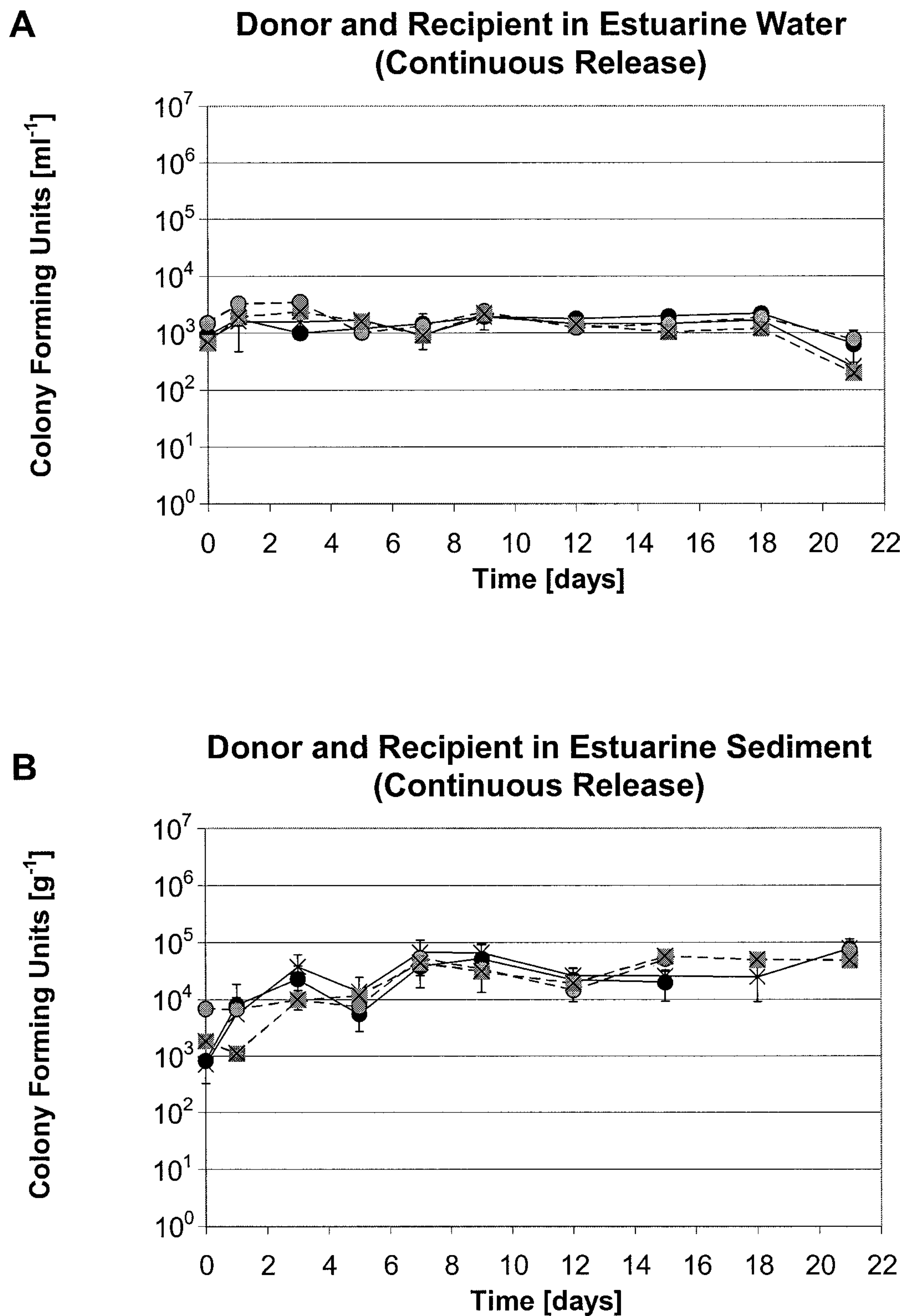

Figure 3. Gene transfer in estuarine microcosms with continuous release of the donor. Bacterial densities of the donor *P. putida* KT2442::mer73-gfp11 and the recipient *P. putida* KT2442::Tc in water and sediment of estuarine microcosms are shown. Both strains were inoculated daily to yield densities of 103 cells/ml. Donor and recipient are represented by a filled circle (-●-) and a cross (-✕-), respectively. Results from control microcosms are shown as well. These were inoculated with the parent strain *P. putida* KT2442 (– -◍- –) and the recipient (– -✕- –). No transconjugants were observed over the duration of the experiment.

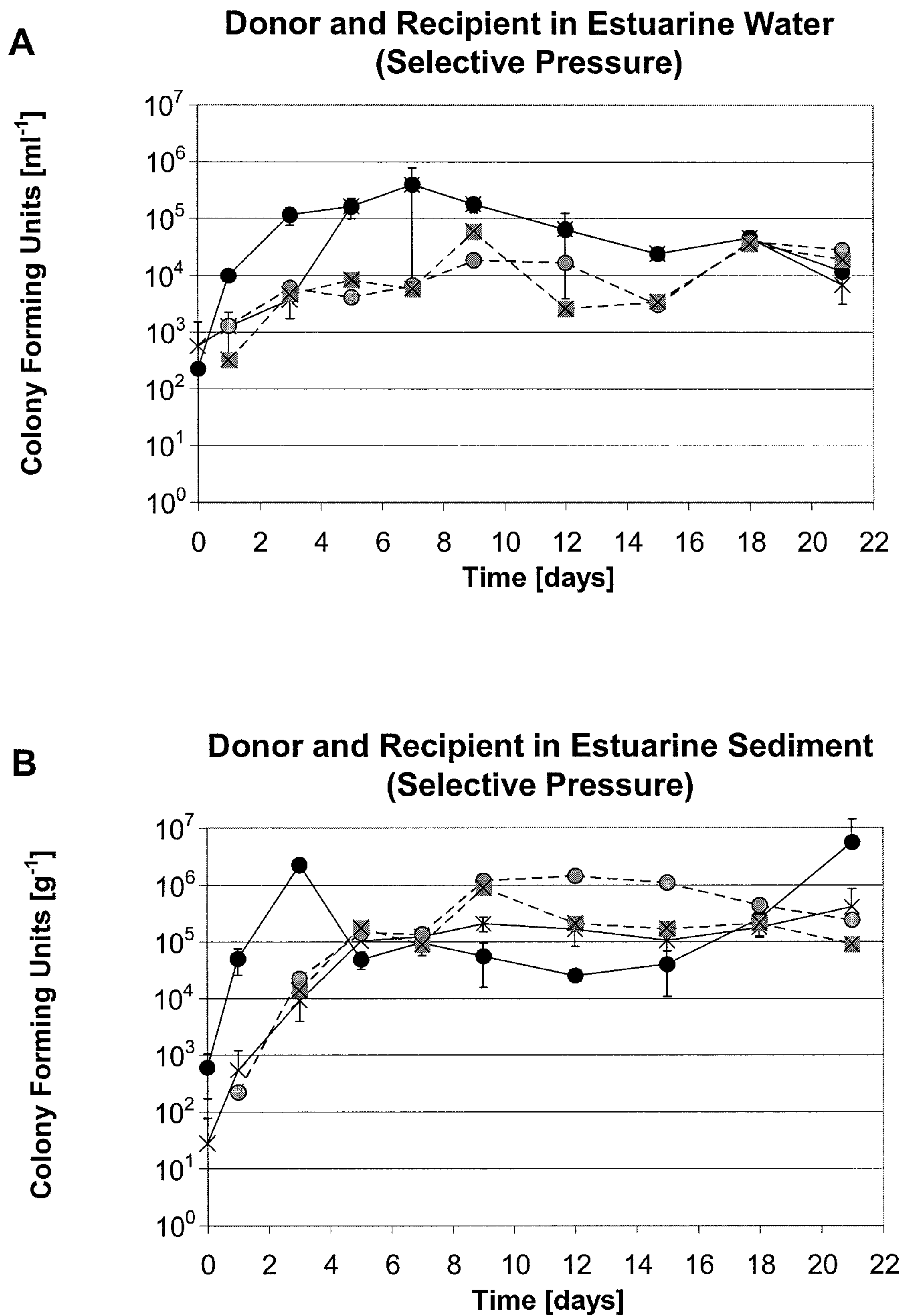

Figure 4. Gene transfer in estuarine microcosms under mercury selective pressure. Bacterial densities of the donor *P. putida* KT2442::mer73-gfp11 and the recipient *P. putida* KT2442::Tc in water and sediment of estuarine microcosms are shown. Both strains were inoculated daily to yield densities of 103 cells/ml. Microcosms were amended with 250 μg/L of phenyl mercuric acetate. Donor and recipient are represented by a filled circle (-●-) and a cross (-✕-), respectively. Results from control microcosms are shown as well. These were inoculated with the parent strain *P. putida* KT2442 (– ◎ – –) and the recipient (– ⊠ – –). No transconjugants were observed over the duration of the experiment.

Discussion

GEMs in Biotechnology and Bioremediation

Recombinant microorganisms have proven very valuable to the biotechnology industry for decades. By now they are indispensable for the production of amino acids, vitamins, alcohol, antibiotics, and secondary metabolites for health and nutrition (Demain, 2000). Furthermore, bacteria have been isolated and engineered to biodegrade or detoxify pollutants (Barkay and Schaefer, 2001). Industrialization has taken its toll by leaving many environmental habitats, especially rivers, polluted and ecosystems severely damaged. Bacteria have, however, evolved a long history of adaptation to adverse conditions, therefore they possess the capacity to cope with pollutants, having already evolved a detoxifying mechanism or developing one under the selective pressure of the toxin (e.g., by expanding the substrate range of an enzyme that is already present) (de Lorenzo, 2001). Recombinant DNA technology has provided a tool for accelerating this process in a rational way. The improvement of microbes that appear promising for bioremediation may be achieved, for example, by creating new metabolic routes, by optimizing substrate routing, by improving turnover rate, or by enhancing process-relevant properties of the microorganism (Pieper and Reineke, 2000; Timmis and Pieper, 1999).

Pseudomonas putida KT2442::mer73 is one example of a GEM that was constructed for improved performance in bioremediation of mercury-contaminated wastewater. While natural bacteria need induction of mercury resistance, this strain reduces mercury at a high rate, independent of the mercury levels in the medium. It is therefore well adapted to cope with fluctuating mercury concentrations (as is typical of industrial bioreactor operations).

Biosafety Considerations

Genetically engineered microorganisms have caused public concern and have been the topic of political discussion (Drobník, 1999). Their ecological impact and the risk of gene transfer to indigenous organisms have been hotly debated. The European legislature has released guidelines for the deliberate release into the environment of genetically modified organisms (Directive 2001/18/EC of the European Parliament and of the Council of March 12, 2001); these demand knowledge of the persistence of the recombinant DNA and its transfer to the indigenous microflora. Other countries, too, have implemented regulations on the use of GEMs. Unfortunately, because of this, studies on the impact of GEMs on microflora (as well as on their competence in bioremediation) are largely impeded and have mainly been restricted to the laboratory [see Sayler and Ripp (2000) for a rare example of a field release study of a GEM]. The fate and survival of GEMs have been studied, for example, by Iwasaki, Uchiyama, and Yagi (1993) and by Leff, McArthur, and Shimkets (1998). The experiments presented here, however, were aimed at studying gene transfer in conditions as close as possible to those found in nature; we will therefore concentrate our discussion on gene transfer.

Horizontal Transfer of Chromosomally Integrated Genes

Gene transfer usually requires the presence of mobile elements (e.g., a plasmid, bacteriophage, or a transposon) that have the ability to take up DNA and release it in another cell in a form that allows translation into protein. Conjugation, transduction, and transposition have been studied and have been found to occur under environmental conditions (Bogdanova et al., 2001; Davison, 1999; Fry and Day, 1990; Lorenz and Wackernagel, 1994). Different conditions, however, may favor gene transfer, depending on the bacterial species and type of transfer mechanism. Chromosomal DNA is generally regarded as stable (Abebe et al., 1997), transfer is assumed to occur too rarely for detection (Sengeløv et al., 2001), and the bacterial genome is viewed to be a safe place for genetic engineering. Conserved chromosomal sequences (such as DNA coding for ribosomal RNA), however, could be relatively frequent targets for homologous recombination and thus serve as natural vehicles for horizontal gene transfer (Arber, 2000; Strätz, Mau, and Timmis, 1996). Furthermore, the uptake of naked DNA (either of chromosomal or plasmid origin), which is liberated if cells lyse, has been shown to be of environmental relevance (Lorenz and Wackernagel, 1994).

Our experiments provided the following possibilities for the dissemination of the chromosomally integrated genes:

The construction of *P. putida* KT2442::mer73 was accomplished by transposon mutagenesis. Although the transposase was not transferred in the course, inner (I) and outer (O) 19 bp Tn5 ends can be recognized by natural broad host range Tn5 transposase variants.

Inoculation into micrococsms must have caused a great number of cells to lyse and thus huge amounts of free DNA available for transformation were delivered into the microcosm. Preliminary results of a quantitative polymerase chain reaction (PCR) approach (Felske et

al., 2001) with stream microcosm samples showed a discrepancy between CFU data and chromosome equivalents by two orders of magnitude (data not shown). Nielsen et al. (2000) found that although chromosomal DNA can persist in soil for weeks, if protected by soil minerals, it may not be transformable for the same length of time. In our experiments, however, cells were inoculated continuously or repeatedly and "fresh" DNA constantly renewed. Although *P. putida* has not been shown to enter a competence state, it can be transformed by chemical treatment or electroporation in the laboratory. Lorenz and Wackernagel (1994) summarize procedures by which DNA can be translocated into cells and they point out that environmental situations may be similar to those procedures. Osmotic shock experienced by the inoculants in our experiments when they were transferred to the microcosms could, for example, have rendered them receptive for the uptake of DNA.

The *mer* operon is widely spread among bacteria and some sequences are highly conserved (e.g., *mer*A; Osborn et al., 1997). By homologous recombination, the *mer* operon (or parts of it) could be exchanged if the GEM would take up a plasmid bearing mercury resistance genes; however, this would in any case remain a silent transfer and we would not have detected it, even if the plasmid transferred to the recipient. In that case we could not discriminate between mercury resistance genes coming from the donor or indigenous bacteria. Since a silent transfer would not result in a detectable phenotype, it would remain without consequence, much less be a risk.

Bacteriophages have been shown to be abundant both in freshwater and marine environments (Miller, 2001). Transduction has been a mechanism for maintaining an otherwise counter-selected phenotype in a continuous culture model (Replicon, Frankfater, and Miller 1995); transduction frequencies in a freshwater environment have been shown to be enhanced in the presence of SPM, where bacteria and bacteriophages can aggregate (Ripp and Miller, 1995). Free phage particles can be assumed to have been abundant also in the microcosms used in this study. These could have infected and lysed the donor, randomly producing *mer*-operon-containing phage particles for which then there would have been a great possibility of transducing the recipient.

Although some of the above scenarios may appear highly speculative, none of them can be ruled out completely. Gene transfer mechanisms operating in the environment may be much more complex than anticipated on the basis of our present knowledge. Thus the experimental proof of lack of gene transfer under the specified conditions, which is provided by our results, cannot be replaced by theoretical considerations, however convincing they may be.

Effect of Selective Pressure on Gene Transfer

Rensing, Newby, and Pepper (2002) review the influence of selective pressure on the horizontal transfer of DNA in soil bacteria. The presence of mercury has, for example, been shown to increase the frequency of mercury resistance plasmids. Smit, Wolters, and van Elsas (1998) demonstrated a greater occurrence of self-transmissible mercury resistance plasmids in mercury-amended soil and suggested that the gene-mobilizing capacity was enhanced as mercury stress was applied. Rasmussen and Sørensen (1998) also found a higher occurrence of mercury resistance and of self-transmissible plasmids in mercury-contaminated marine sediment as compared to unpolluted sediment. The positive effect of selective pressure on the emergence of transconjugants could also be shown for phenoxyacetic acid and the *tfd*A-bearing plasmid pRO103 (coding for a 2,4-dichlorophenoxyacetic acid dioxygenase) (de Lipthay, Barkay, and Sørensen, 2001).

These studies show that the application of a mercury selective pressure can increase the frequency of horizontal transfer of the mercury resistance genes, and that it was hence an adequate step to promote transconjugants in our experiments. Despite this fact, gene transfer could not be observed in our experiments.

Effect of Cell Density

It has been recognized that cell densities play an important role in horizontal gene transfer. It is also conceivable that for a gene transfer mechanism that requires cell-cell contact, such as conjugation, the chance for horizontal gene exchange would increase with increasing cell densities. For filter mating in the laboratory, for example, fresh overnight cultures are commonly used and concentrated to increase the cell density on the filter. Accordingly, Ravatn, Zehnder, and van der Meer (1998) found decreasing transconjugant frequencies with lower cell densities. Fry and Day (1990) admonish against using low initial cell densities or too few donors because conjugal transfer frequencies (in the epilithon) declined rapidly if densities were less than 2×10^5 CFU/cm^2. They found the optimal donor:recipient ratio to be between 1:60 and 16:1. Donor densities used to study conjugal transfer to indigenous aquatic microorganisms or to bacteria within a pilot-scale percolating filter bed were approximately 10^5

or 10^6 CFU/ml (Ashelford, Learner, and Fry, 2001; Barkay, Liebert, and Gillman, 1993). For our experiments, this means that gene transfer would be expected in the more densely populated sediment rather than in the water column. If the continuous release of lower cell densities did not result in gene transfer, a cell shock with high cell density might possibly induce it. Neither approach, however, resulted in the detection of transconjugants.

Transformation is dependent on cell-DNA contact frequencies, which in turn are also increased with higher cell densities and DNA concentrations. Other parameters, however, may play a more crucial role in the transformation process, for example, the half-life of free DNA in the environment in question and the competence state of the recipient. These factors are reviewed by Lorenz and Wackernagel (1994). As already stated above, in our experiments we provided a constant renewal of fresh DNA molecules from lysed inoculants, of which surely some would have been transformable even if a great portion was degraded by DNase.

Role of SPM

In river water, mercury is predominantly adsorbed to SPM, although colloidal mercury may play a role during high-flow conditions (Choe, Gill, and Lehmann, 2003; Lead et al., 1999). SPM also serves as a matrix for bacteria to attach to and grow on (Böckelmann et al., 2002). The effect of the bacterial biofilms on the transformation of mercury species has been investigated (Ebinghaus and Wilken, 1993). The SPM acts as a vector, transporting mercury horizontally (downstream) as well as vertically (sedimentation), forming the top sediment layer in zones of low-flow velocity of the river. Where natural bacteria develop dense populations—as on SPM or in sediments—conjugal transfer of plasmids will most likely take place (Fry and Day, 1990; Leff, McArthur, and Shimkets, 1992). Horizontal conjugal transfer of heavy metal resistance genes has been reported between bacteria from diverse genera, including gram-negative and gram-positive bacteria, not only under laboratory conditions (e.g., in plate matings), but also under the complex conditions of an activated sludge ecosystem (Dong et al., 1998). For conjugal transfer of the chromosomally integrated *mer* operon to occur, its mobilization into a conjugative plasmid would be required. Both recombination events and transposition play major roles in the mobilization of chromosomal genes. Integration of DNA fragments into the chromosome has, for example, been described for *Acinetobacter calcoaceticus* (Palmen and Hellingwerf, 1997).

Conclusion

While the dissemination of genes from GEMs is regarded as an unpredictable risk by some (Ashelford, Learner, and Fry, 2001), for others it could represent a valuable method of bioremediation in that it provides a wide variety of microorganisms with the detoxifying or degrading capabilities in question (Barkay, Liebert, and Gillman, 1993; de Lipthay, Barkay, and Sørensen, 2001). Gene transfer from *P. putida* KT2442::mer73 should not be viewed as risky, because bacterial mercury resistance genes are ubiquitous and can be found even in noncontaminated environments (Osborn et al., 1997). The dissemination of the genes in mercury-contaminated regions would aid in biological mercury transformation, and the site would profit with regard to pollution severity.

We believe that our experiments have provided conditions under which the probability for horizontal gene transfer was greatly enhanced. The fact that we could still not detect any suggests that construction of the mercury-reducing GEM has yielded a strain with stable integration of engineered genes that are not readily disseminated. On the basis of these experiments, the strain *P. putida* KT2442::mer73 can be regarded as safe in applications involving release into the environment.

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