Effect of mercury addition on plasmid incidence and gene mobilizing capacity in bulk soil

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Abstract

Two methods of exogenous plasmid isolation were used to evaluate and compare the plasmid incidence and gene mobilizing capacity of eight soils before and after the addition of mercury. Biparental matings (BM) were performed using \textit{Pseudomonas putida} UWC1 as recipient to obtain conjugative mercury resistance (Hg\textsuperscript{R}) plasmids. To obtain mobilizing plasmids, the transfer of the IncQ vector pMOL187 from \textit{Escherichia coli} into \textit{Ralstonia eutropha} was selected in triparental matings (TM). The numbers of donor, helper and recipient cells used in the matings were kept the same for each soil sample, which provided a way to compare plasmid incidence and gene mobilizing capacity on the basis of the numbers of transconjugants obtained. Using BM prior to mercury addition, plasmids that confer Hg\textsuperscript{R} were obtained only from 1 of 8 soils. However, following the addition of mercury to these soils, Hg\textsuperscript{R} transconjugants were obtained from 5 of the 8 soils, and among these broad (BHR) and narrow-host-range (NHR) plasmids were found at variable ratios. Similarly, these same five soils were the only ones to yield mobilizing plasmids by using TM; all of which were found to confer Hg\textsuperscript{R} and to be BHR plasmids. This suggests that Hg\textsuperscript{R} plasmids were the main cause of the increased mobilizing capacity observed in the mercury treated soils. Notably, there were three soils in which no plasmids that confer mercury resistance were obtained although the soils had at least $10^5$ Hg\textsuperscript{R} cfu g soil\textsuperscript{-1}, indicating that resistance to mercury may not be encoded on plasmids that could be transferred to the recipients used. Plasmids from 177 transconjugants obtained by BM and TM could be classified into 20 groups based on size and restriction fragment patterns, and none belonged to known classes of BHR plasmids. This indicates that there are diverse replicons capable of mediating the dissemination of mercury resistance.

Keywords: Soil; Mercury amendment; Community shift; Broad-host-range plasmid

1. Introduction

Previous studies have demonstrated the importance of horizontal gene transfer on the evolution and diversification of bacteria [1,2]. Direct (biparental) and triparental transfer of plasmids, as well as retromobilization of plasmids, are thought to be important to this process [3–5]. Furthermore, certain plasmids can integrate into bacterial chromosomes and may mobilize chromosomal DNA into another bacterium when they are subsequently transferred.
Transferred plasmids and chromosomally encoded loci may confer antibiotic or heavy metal resistance, the ability to metabolize recalcitrant compounds, as well as other phenotypic traits that may provide a selective advantage for the cell and lead to their increased survival and growth. For these reasons, an understanding of horizontal gene transfer mediated by plasmids is important in order to understand the risk associated with the release of recombinant DNA into the environment [6] regardless of whether the recombinant genes are encoded on plasmids or integrated into chromosomes.

Several approaches have been used to assess the frequency of conjugal transfer of plasmids in the environment. To measure plasmid transfer to indigenous bacteria or plasmid transfer frequencies during changing physiochemical conditions investigators have introduced donor cells, or donor and recipient cells into microcosms [7-14]. Others have used exogenous plasmid isolation [15] wherein a recipient strain [16,17], or a recipient strain together with a donor strain with a non-conjugative mobilizable (Tra$^-$ Mob$^+$) plasmid [18] are introduced to the microcosm or environment to assess the presence of transferable and mobilizing plasmids. Environmental parameters that inhibit conjugation can be overcome if the latter approach is done as matings between mixtures of bacteria on agar [19-21], which may allow a much larger portion of conjugative and mobilizing plasmids to be obtained.

A group of plasmids predicted to be of particular importance in connection with horizontal gene dissemination are broad-host-range (BHR) plasmids. While the definition of BHR plasmids is the subject of debate, it has been proposed that BHR plasmids can be defined as those plasmids able to transfer and replicate in at least two distinct phylogenetic groups of bacteria (such as different subdivisions of the Proteobacteria [22]). Recent studies indicate that most BHR plasmids isolated from environmental samples have little or no homology to known plasmid incompatibility groups [23-26], suggesting that BHR plasmids are more diverse than previously recognized. Thus an assessment of their occurrence requires that functional assays are done to determine their host range after they have been isolated by any of several methods. The exogenous plasmid isolation method of Top et al. [27] is useful for this purpose wherein a triparental mating is done using a γ and β member of the Proteobacteria as donor and recipient strains. Plasmids from the bacterial community captured in the recipient bacteria together with the Tra$^-$ Mob$^+$ vector under selection, were thus expected to have a broad transfer range.

In the present study the plasmid incidence and gene mobilizing capacity of several soils before and after treatment with mercury were compared in a quantitative way. This was done using biparental and triparental matings that were performed in parallel using the same numbers of helper, donor and recipient cells. The different plasmid types obtained were characterized with respect to their host range and their incompatibility group.

2. Materials and methods

2.1. Bacterial strains, plasmids and culturing conditions

The plasmids and bacteria used in this study are listed in Table 1. Luria-Bertani broth (LB, [28]) amended with an appropriate selecting compound was used for growing bacterial cultures. Recipient bacteria for mating experiments were grown with 200 μg ml$^{-1}$ rifampin (Rif), whereas the donor strain in triparental matings (TM), Escherichia coli CM1308, was grown with 50 μg ml$^{-1}$ ampicillin to maintain selection for the plasmid pMOL187 (Amp$^r$). When growing bacteria with mercury resistance (Hg$^{2+}$) plasmids for mating purposes, none or a minor amount of HgCl$_2$ (0.5 μg ml$^{-1}$) was used in the medium. The broth cultures were shaken on a rotary platform at approximately 200 rpm. Incubation temperature was 37°C for E. coli strains and 28°C for the other bacteria. Matings were allowed to take place on LA plates (LB with 1.5% Bacto agar). Cycloheximide (chm, 10 μg ml$^{-1}$) was added to the plate media used in the soil experiments to inhibit fungal growth.

2.2. Soil samples and soil characteristics

Samples from seven agricultural soils and one sample from beech forest soil were collected from sites in southern Norway during the autumn of
1995. The soil samples were collected from the upper soil layers (20 cm), and stored in plastic bags at room temperature for two to three weeks prior to the start of the experiments. The soils were analyzed with respect to pH, moisture content, field capacity and organic content (Table 2). Prior to the experiment, 350 g of sieved (2-mm-mesh) soils were prepared in 1.5-l glass jars. Mercury was added to the soils in 5-ml portions of a HgCl2 solution to obtain a final concentration of 25 μg HgCl2 per gram soil wet weight.

2.3. Preparation of bacterial fractions from the soils

Soil samples (5 g) were diluted 10-fold in sterile phosphate buffered saline solution (PBS; 10 mM Na2HPO4·2H2O and 10 mM NaH2PO4·2H2O, pH 7.5) in 50-ml Falcon tubes. The tubes were shaken for 30 s with a Whirlmixer before sonication for 5×20 s submerged in water (Bandelin, Sourex, Rapid). Unsuspended soil particles were collected by centrifugation in a Sorvall SS34 rotor at 1000 rpm for 30 min, and the supernatant with the bacterial cells was transferred into new tubes and centrifuged further at 10000 rpm for 10 min. The supernatant was then discarded and the cell pellet with the bacteria resuspended in 2 ml PBS. To determine the total cell number in the fraction, a 10-μl sample was diluted in 1 ml sterile PBS and filtered on black Nucleopore filters (0.2 μm, Nucleopore, Pleasanton, CA, USA). The preparations were covered with DAPI (4’-6-diamidino-2-phenylindole, 1 μg ml−1) [29] for 5 min and washed in sterile PBS, and the stained bacteria were counted using an epifluorescence microscope (EFM, Nikon).

2.4. Examination of colony forming units (cfu) and colony morphology types

Total cfu in the soils were determined by plating 0.1 ml of a 10-fold dilution (in sterile PBS) of the bacterial fractions on R2A (Difco) medium supplemented with 10 μg ml−1 chm. The cfu of mercury resistant bacteria (HgR bacteria) were determined on R2A medium containing 25 μg ml−1 HgCl2 and 10 μg ml−1 chm. The colonies on both media were assigned to different colony morphology types, based on visual observation (color, shape and surface appearance) of the colonies. The analyses were done before mercury addition and at 6, 12 and 56 days after mercury amendment. The plates were incubated at 20°C and colonies were counted and studied after three days of incubation and again seven days later to include slower growing bacteria.

2.5. Examination of plasmid incidence and gene mobilizing capacity

The bacterial fractions from each soil were examined for plasmid incidence and gene mobilizing capacity before and 12 days after mercury addition using two methods of exogenous plasmid isolation. The two methods, bi- and tri-parental mating (BM and TM), were performed in parallel in a quantitative way using plate matings. For BM the mating mixtures were prepared from 4×109 cells of the soil bacterial fractions and 4×108 of PBS washed Pseudomonas putida UWC1 cells suspended in 1 ml (1:10) LB. The mating mixture for TM was prepared with similar cell numbers, but Ralstonia eutropha AE815 (Table 1) was used as recipient strain and additionally 4×109 of PBS washed donor cells in 1 ml 1:10 LB were added. The donor strain, E. coli CM1308, harbors the non-conjugative vector pMOL187, but cannot express the czc genes (Co2+, Zn2+, and Cd2+) encoded by the plasmid. When the genes are transferred to R. eutropha AE815 the heavy metal resistance is expressed in this strain. The mating mixtures were poured onto LA plates, left open to dry in a laminar flow hood, and incubated for 18 h at 25°C to allow conjugation to occur. The cells were harvested by suspending the cell layer in 5 ml of sterile PBS. The cell suspensions were serially diluted in PBS, and 0.1-ml samples were plated on the selective media to select for transconjugants. For BM this was LA with 200 μg ml−1 Rif, 25 μg ml−1 HgCl2 and 10 μg ml−1 chm, which allowed selection for P. putida (RifR) containing a HgR plasmid. The selective medium for TM was Tris mineral medium [30] supplemented with azelaic acid (0.2%), Rif (200 μg ml−1), Zn2+ (2 mM) and chm (10 μg ml−1). These plates allowed the selection of zinc resistant R. eutropha (RifR) transconjugants containing plasmid pMOL187. LA plates supplemented with 200 μg ml−1 Rif and 10 μg ml−1 chm were used to assess the total number of recipient bacteria and
the background in the bacterial fractions. Transconjugant and recipient selective plates were incubated at 28°C for 2 days for the *P. putida* UWC1 transconjugants and for at least 3 days for *R. eutropha* AE815 transconjugants.

2.6. Screening and confirmation of transconjugants

From the transconjugant plates used in the exogenous plasmid isolation, 140 colonies from BM and 170 colonies from TM were screened for plasmids by a modified Eckhardt method [31]. The randomly picked colonies from BM were streaked onto LA plates containing 50 μg ml⁻¹ HgCl₂, whereas the TM transconjugants were streaked on LA plates without any supplement. The bacteria were grown overnight at 28°C. With a toothpick, the fresh cell material was resuspended in 30 ml lysis buffer (50 mM Tris-HCl, pH 8, 10 mM EDTA, pH 8, 10 μg ml⁻¹ lysozyme, 8 μg ml⁻¹ RNAse, 0.04% (w/v) bromophenol blue and 6.9% (w/v) sucrose). After a freeze-thawing step the cells were loaded into the wells of a 0.7% agarose gel with 1% SDS [24]. One μl 5 μg ml⁻¹ protease were added into the wells and the enzyme was allowed to work for 15 min, then the gel was run at 8 mA for 2 h and 45 mA for 4 h. The plasmids of *E. coli* V517 and *E. coli* K12 (Table 1) were used as plasmid size markers. Fluorescent antibody staining was used to identify 140 of the *P. putida* UWC1 transconjugants obtained from biparental isolation. The polyclonal antibody against UWC1 was raised in rabbit and prepared as described by Enger et al. [32]. Formaldehyde treated cells were heat fixed on object slides (12 wells, 4 mm; HTC Super Cured; Flow Laboratories, Scotland). The object slides were covered with the anti-PPUWC1 antiserum (diluted 1:5000 in PBS with 1% bovine serum albumin) for 20 min, washed in PBS, and incubated with fluorescein-isothiocyanate (FITC)-labelled secondary antibodies for 30 min. After a final PBS wash, the preparations were dried and cover slips mounted with immersion oil. Examination was done in EFM using a FITC filter package. Transconjugants from TM were examined by REP-PCR and the patterns were compared with that of *R. eutropha* AE815. The REP-PCR method was performed as described by de Bruijn [33] except that 1 μl freeze-thawed cells was mixed with 24 μl of the PCR mixture.

2.7. Isolation of plasmid DNA and restriction analysis

The plasmids to be characterized were isolated by a modified method of Birnboim and Doly [34], with the main changes as described by Valla et al. [35]. Cells were grown overnight in 10 ml LB and harvested by centrifugation at 3000×g for 5 min and resuspended in 1.2 ml SET buffer (25% sucrose, 50 mM EDTA, 50 mM Tris-HCl, pH 8). A 2.8-ml 1:1 mixture of 0.4 N NaOH and 2% SDS was added and the tubes were kept on ice for 10 min. Two ml 3 M Na-acetate (pH 4.8) was added and the solution was carefully mixed and incubated further on ice for 20 min. Then the tubes were centrifuged at 6000×g and the supernatant was transferred to new tubes. One volume of isopropanol (−20°C) was added, and the tubes were incubated at −20°C for 30 min before the plasmid DNA was precipitated by centrifugation at 6000×g for 20 min. The pellet was dried, dissolved in 500 μl distilled H₂O and transferred to an Eppendorf tube. Then 170 μl of 10 N NH₄-acetate was added and the tubes were kept on ice for 20 min before centrifugation for 10 min at 6000×g. The supernatant was transferred into new tubes together with 700 μl isopropanol (−20°C). After 30 min at −20°C the plasmids were precipitated (20 min at 6000×g), and the pellet washed in 70% cold ethanol, dried, and redissolved in 40 μl distilled H₂O. *EcoRI* restriction digests of the plasmids were performed as recommended by the manufacturer (Boehringer Mannheim). The fingerprint patterns were revealed by electrophoresis in a 1.2% agarose gel run at 30 V overnight.

2.8. Replicon probing

The DNA probes used for replicon typing were repPₓ, repPₘ, repN, repW, repQ and repU, representing the best known incompatibility groups of broad-host-range plasmids. The probes were prepared by digesting the corresponding vectors with the appropriate restriction enzymes, as described by Couturier et al. [36] and Smith and Thomas [37]. The DNA was extracted from the agarose gel using the GeneClean II kit (BIO 101), and labelled with a digoxigenin d-UTP DNA-labelling kit (Boehringer Mannheim, Indianapolis, IN, USA) according to the manufacturer’s instructions. The probes are as-
associated with the replication or partition function of the plasmids and have been shown to hybridize only with plasmids belonging to the corresponding incompatibility groups. Plasmid DNA to be examined was blotted on nylon membranes (Hybond N; Amersham) and fixed on a UV illuminator. Hybridizations were done under stringent hybridization conditions (5×SSC, 0.1% N-lauroyl sarcosine, 0.02% SDS, 50% formamide, 5% blocking powder) at 62°C allowing only sequences with >90% DNA homology to be detected. Signal detection was done with Boehringer Mannheim’s DNA chemiluminescence detection kit following the manufacturer’s instructions.

2.9. Host range studies

The different plasmid types captured in the exogenous isolations were examined for their ability to transfer into bacterial strains from different phylogenetic subdivisions. Plasmids captured in *P. putida* UWC1 were initially transferred into *E. coli* HB101 (Str<sup>r</sup>, Rif<sup>r</sup>, Leu<sup>−</sup>) selecting for mercury (50 μg ml<sup>−1</sup>) and streptomycin (200 μg ml<sup>−1</sup>) resistance at 37°C. The plasmids obtained in *R. eutropha* AE 815 were transferred into *E. coli* CM330 (Met<sup>−</sup>) as described below. As a negative control on the transfer, the auxotrophic *E. coli* transconjugants were streaked on Vogel and Bonner medium [38] without methionine. The Rif<sup>r</sup> strains *Agrobacterium tumefaciens* UBAPF2 (α-subdivision), *P. putida* UWC1 (γ-subdivision), *R. eutropha* AE815 (β-subdivision) and *Comamonas acidovorans* (β-subdivision) were the recipient bacteria used in the host range study. All examinations were performed using patch matings. Droplets of 5 μl donor and 5 μl recipient bacteria from freshly grown cultures were mixed on LA plates. *E. coli* CM330 with the Hg<sup>II</sup> plasmid pJP4 (IncP) in the donor strain was used as a positive control. After 18 h of incubation at 28°C, the cells were resuspended in PBS and plated on a selective medium for transconjugants (LA with 200 μg ml<sup>−1</sup> Rif and 50 μg ml<sup>−1</sup> HgCl<sub>2</sub>) and recipients (LA with 200 μg ml<sup>−1</sup> Rif). Donors and recipients were examined separately for controls, and transconjugants were confirmed to contain the plasmids by the Eckhardt screening method.

2.10. IncQ mobilization and mercury resistance properties

The selected plasmids captured through BM were examined for their ability to mobilize the IncQ vector pMOL187. This was done in triparental patch matings using the same donor and recipient system as in TM. Mostly *E. coli* HB101 (Str<sup>r</sup>, Rif<sup>r</sup>) was used as host for the plasmids, but plasmids not transferring to HB101 were examined in their original recipient, *P. putida* UWC1. Transconjugants were screened for plasmids by the Eckhardt method. Also plasmids captured in TM were examined for their ability to mobilize pMOL187 (Amp<sup>r</sup>). This was done in biparental matings between the original *R. eutropha* transconjugants and *E. coli* CM330 using selective LA plates (50 μg ampicilline ml<sup>−1</sup>) and incubation at 42°C for donor counter selection. Transconjugants were screened by the Eckhardt method. The plasmids captured via TM were also examined for possible Hg<sup>II</sup> determinants using the same mating system, but selecting for mercury (50 μg ml<sup>−1</sup>).

3. Results

3.1. Effect of mercury on bacterial numbers and diversity

The effect of mercury addition on the total number of viable and Hg<sup>II</sup> bacteria was determined before the addition of Hg, as well as 6, 12 and 56 days thereafter. The numbers of viable bacteria in the soils were initially 10<sup>6</sup>–10<sup>7</sup> cfu g<sup>−1</sup> and only the StendS soil had Hg<sup>II</sup> bacteria above the detection limit (10 cfu g<sup>−1</sup>). Following mercury addition, the numbers of Hg<sup>II</sup> bacteria initially increased to 10<sup>5</sup>–10<sup>7</sup> cfu g<sup>−1</sup>, but tended to decrease thereafter, whereas the total numbers of viable bacteria were largely unaffected (Table 3). One exception was the Groningen soil which showed a 1000-fold increase in the number of viable bacteria between day 6 (7.5×10<sup>6</sup> cfu g<sup>−1</sup>) and day 12 (2.3×10<sup>7</sup> cfu g<sup>−1</sup>) while during the same period there was a 1000-fold decrease in Hg<sup>II</sup> bacteria (4.4×10<sup>6</sup>–1.9×10<sup>3</sup> cfu g<sup>−1</sup>). The diversity of numerically dominant bacteria (based on col-
ony morphology) appeared to decrease following the addition of Hg. There were on average 13 morphotypes before mercury addition, but only 3–4 morphotypes observed 56 days after the addition of Hg (Table 4). These data indicate that significant changes in community structure occurred in response to the addition of mercury.

3.2. Effect of mercury on the incidence of Hg$^R$ and mobilizing plasmids in soil

The presence of conjugative Hg$^R$ and IncQ mobilizing plasmids in eight soils before and 12 days after mercury addition was examined by using biparental and triparental mating procedures (BM and TM; Table 5). The total number of recipient cells recovered ranged from 5×10^9 to 5×10^10 per ml mating mixture. Prior to the addition of mercury, Hg$^R$ P. putida UWC1 transconjugants were only obtained from the StendS soil. Similarly, BHR plasmids with the ability to mobilize the IncQ vector pMOL187 were only obtained in two soils (StendO and Garpestad) before mercury addition. A clear effect of mercury addition on the number of transconjugants in BM and TM was observed in five out of the eight soils wherein at least 10^2 transconjugants were obtained per ml of mating mixture (Table 5). These data show that contamination with mercury enhanced not only the level of conjugative Hg$^R$ plasmids, but also the gene mobilizing potential in five of the eight soils studied.

### Table 2

<table>
<thead>
<tr>
<th>Soil</th>
<th>Characteristics</th>
<th>pH</th>
<th>Total counta per g soil wet weight</th>
<th>Dry weight (%)</th>
<th>% Organic matter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seim Beechwood</td>
<td></td>
<td>2.9</td>
<td>9.2×10^9</td>
<td>20</td>
<td>76</td>
</tr>
<tr>
<td>Grøningen Grassland, unmanurated</td>
<td></td>
<td>3.4</td>
<td>1.6×10^10</td>
<td>64</td>
<td>7</td>
</tr>
<tr>
<td>Galdal Field: Potatoes, grassland</td>
<td></td>
<td>4.4</td>
<td>1.1×10^10</td>
<td>78</td>
<td>9</td>
</tr>
<tr>
<td>Trysil Meadow</td>
<td></td>
<td>5.2</td>
<td>1.2×10^10</td>
<td>76</td>
<td>9</td>
</tr>
<tr>
<td>StendO Grassland</td>
<td></td>
<td>5.7</td>
<td>1.4×10^10</td>
<td>44</td>
<td>54</td>
</tr>
<tr>
<td>StendS Field: Cereal, vegetables</td>
<td></td>
<td>6.0</td>
<td>1.0×10^10</td>
<td>74</td>
<td>9</td>
</tr>
<tr>
<td>Garpestad Field: Cereal, vegetables</td>
<td></td>
<td>4.5</td>
<td>1.1×10^10</td>
<td>65</td>
<td>18</td>
</tr>
<tr>
<td>Ås Field: Cereal</td>
<td></td>
<td>6.0</td>
<td>2.7×10^10</td>
<td>83</td>
<td>4</td>
</tr>
</tbody>
</table>

aKCl method.
bFluorescence microscopy of DAPI stained preparates.cExpressed on soil dry weight.
### Table 3
Effect of mercury addition on total and Hg\textsuperscript{II} bacterial counts in eight soils\textsuperscript{a}

<table>
<thead>
<tr>
<th>Soil</th>
<th>Total cfu g\textsuperscript{-1} soil wet weight\textsuperscript{b}</th>
<th>Hg-cfu g\textsuperscript{-1} soil wet weight\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before\textsuperscript{d}</td>
<td>Day 6 \textsuperscript{e}</td>
</tr>
<tr>
<td>Seim</td>
<td>1.5 × 10\textsuperscript{7}</td>
<td>2.0 × 10\textsuperscript{6}</td>
</tr>
<tr>
<td>Groningen</td>
<td>4.7 × 10\textsuperscript{7}</td>
<td>9.0 × 10\textsuperscript{6}</td>
</tr>
<tr>
<td>Galdal</td>
<td>4.2 × 10\textsuperscript{7}</td>
<td>1.3 × 10\textsuperscript{6}</td>
</tr>
<tr>
<td>Trysil</td>
<td>8.5 × 10\textsuperscript{6}</td>
<td>4.5 × 10\textsuperscript{7}</td>
</tr>
<tr>
<td>Galdal</td>
<td>9.0 × 10\textsuperscript{6}</td>
<td>4.2 × 10\textsuperscript{7}</td>
</tr>
<tr>
<td>StendO</td>
<td>1.3 × 10\textsuperscript{6}</td>
<td>1.9 × 10\textsuperscript{6}</td>
</tr>
<tr>
<td>StendS</td>
<td>8.5 × 10\textsuperscript{6}</td>
<td>1.0 × 10\textsuperscript{6}</td>
</tr>
<tr>
<td>Garpestad</td>
<td>4.5 × 10\textsuperscript{7}</td>
<td>2.1 × 10\textsuperscript{7}</td>
</tr>
<tr>
<td>Ås</td>
<td>3.6 × 10\textsuperscript{6}</td>
<td>1.4 × 10\textsuperscript{7}</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Measured from the bacterial fractions, prepared as described in Section 2.

\textsuperscript{b}Total cfu, determined on R2A medium.

\textsuperscript{c}Hg-cfu, determined on R2A-Hg plates.

\textsuperscript{d}Before, before mercury addition.

\textsuperscript{e}Days after mercury addition.

\textsuperscript{f}ND, not determined.

### 3.3. Transconjugant screening and restriction analysis of selected plasmids

No false positive transconjugants were detected using either BM or TM. In BM, a total of 140 transconjugants were examined and all were found to contain at least one plasmid, whereas all 175 transconjugants from TM contained pMOL187. Thirty-seven of the latter transconjugants contained, in addition to pMOL187, a second plasmid, which should be the mobilizing plasmid. The plasmids obtained from BM and TM were classified based on differences in their electrophoretic mobility and EcoRI restriction fragment patterns (Fig. 1A,B). In a few cases, plasmids that migrated differently in the Eckhardt gel had similar restriction digest patterns. Thus, co-integrates or different configurations of one and the same plasmid occurred in the Eckhardt gels. Based on the combination of both analyses, we found that there were 15 different types of plasmids obtained by BM and 7 types obtained by TM. Their distribution among the soils and time points of capture is shown in Fig. 1A and B. By assuming that plasmids from the same matings were similar when they had the same electrophoretic mobility in the Eckhardt gel, it was possible to estimate the fre-

### Table 4
Effect of HgCl\textsubscript{2} addition on the number of colony morphotypes in eight soils

<table>
<thead>
<tr>
<th>Soil</th>
<th>On R2A plates</th>
<th>On R2A-Hg plates\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before\textsuperscript{b}</td>
<td>Day 6</td>
</tr>
<tr>
<td>Seim</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>Groningen</td>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td>Galdal</td>
<td>11</td>
<td>7</td>
</tr>
<tr>
<td>Trysil</td>
<td>19</td>
<td>9</td>
</tr>
<tr>
<td>StendO</td>
<td>13</td>
<td>14</td>
</tr>
<tr>
<td>StendS</td>
<td>16</td>
<td>11</td>
</tr>
<tr>
<td>Garpestad</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>Ås</td>
<td>11</td>
<td>10</td>
</tr>
</tbody>
</table>

\textsuperscript{a}R2A-Hg, R2A medium supplemented with 25 mg ml\textsuperscript{-1} HgCl\textsubscript{2}.

\textsuperscript{b}Before, before mercury addition.

\textsuperscript{c}ND, not determined.

\textsuperscript{d}Days after mercury addition.
frequency of occurrence of the various plasmid types (Fig. 1A). The mean size of the different plasmids captured with the two methods was estimated to be 133 kb in BM and 100 kb in TM (Table 6).

3.4. Host range of the captured plasmids

To determine the host range of the plasmid types obtained, representative plasmids were transferred to E. coli HB101 which was subsequently used as a donor in matings with A. tumefaciens UBAPF2, R. eutropha AE815 and Comamonas acidovorans EEZ23. Three of the plasmid types captured in BM did not transfer into E. coli HB101 and were not studied further. Of the remaining 12 plasmid types, only 5 could be transferred to one or more strains belonging to a different phylogenetic subdivision (Table 6) and therefore shown to be BHR plasmids. As expected, all the plasmids from TM that were examined were shown to be BHR plasmids (Table 6). Based on the incidence of various plasmid types (Fig. 1A), we found that 6, 88 and 100% of the plasmids from the StendS, Ås and StendO soils, respectively, were BHR plasmids. In contrast, all of the plasmids obtained from Garpestad and Trysil were narrow-host-range (NHR) plasmids. Thus, there was no obvious pattern in the distribution of BHR and NHR plasmids obtained from the five soils.

The plasmid types obtained from the StendS soil through BM before and after Hg addition were compared. Such an assessment was only possible for this soil, as it was the only soil from which a high number of transconjugants (1.2 $\times$ 10$^3$ cfu ml$^{-1}$ mating mixture) was obtained also prior to Hg amendment. Twenty-six transconjugant colonies obtained before and 17 colonies obtained after mercury addition were examined, and a clear shift in plasmid types was observed. Two plasmid types, both found to be NHR, were obtained before mercury addition. One of these, pAKD5, which was initially found in 4% of the transconjugants, constituted 12% of the plasmids after Hg addition. Interestingly, the second plasmid type, pAKD6, was found in 96% of the transconjugants before Hg addition, but was not observed in any of the transconjugants after mercury treatment. Instead three new plasmid types (pAKD4 (6%), pAKD7 (53%), and pAKD8 (29%)) were obtained after Hg addition, of which only one (pAKD4) was a BHR plasmid, found in only 6% of the transconjugants. Thus, amendment of this

<table>
<thead>
<tr>
<th>Soil</th>
<th>BM P. putida UW1 transconj. (cfu ml$^{-1}$)</th>
<th>TM R. eutropha AE815 transconj. (cfu ml$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seim</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Groningen</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Galdal</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Trysil</td>
<td>4.6 $\times$ 10$^2$</td>
<td>2.1 $\times$ 10$^4$</td>
</tr>
<tr>
<td>StendO</td>
<td>1.2 $\times$ 10$^3$</td>
<td>5.0 $\times$ 10$^3$</td>
</tr>
<tr>
<td>StendS</td>
<td>2.7 $\times$ 10$^3$</td>
<td>1.3 $\times$ 10$^3$</td>
</tr>
<tr>
<td>Garpestad</td>
<td>6.6 $\times$ 10$^3$</td>
<td>5.2 $\times$ 10$^2$</td>
</tr>
<tr>
<td>Ås</td>
<td>4.2 $\times$ 10$^3$</td>
<td>9.5 $\times$ 10$^3$</td>
</tr>
</tbody>
</table>

a Colony forming units per ml bacterial fraction.

b — Values below detection limit (10 cfu ml$^{-1}$ mating mixture).

Fig. 1. Distribution of the different plasmid types obtained by bi- and tri-parental mating in eight soils before and after mercury addition. A: The plasmids shown in an Eckhardt gel. Above every line the number of times each plasmid was observed is indicated. B: The EcoRI restriction pattern of the plasmids. The plasmids pAKD2 and pAKD21 were not isolated successfully from the recipient bacteria. Plasmids obtained by TM are marked with bold letters, whereas plasmids from BM are in normal letters. Underlined names are plasmids obtained before mercury addition. E. coli K12: Escherichia coli K12 with two reference plasmids (212 and 159 kb). ▼ indicates weak plasmid bands.
soil with mercury increased the diversity of Hg\textsuperscript{II} plasmids found among the transconjugants, and BHR plasmids were not dominant.

Replicon typing by hybridization of the isolated plasmids with probes to origins of broad-host-range plasmid replication showed that none had homology to IncP\textsubscript{K}, IncP\textsubscript{L}, IncW, IncN, IncQ or IncU plasmids (data not shown). This indicates that the captured BHR plasmids do not belong to the commonly known incompatibility groups of BHR plasmids.

### 3.5. Mobilization and Hg\textsuperscript{II} phenotypes of captured plasmids

The plasmids captured via BM and TM were examined for their ability to mobilize the IncQ vector pMOL187. Four of the 15 different plasmid types captured in BM could mobilize the IncQ plasmid pMOL187 from \textit{E. coli} CM1308 to \textit{R. eutropha} AE815 (Table 6). Interestingly, these four plasmid types were found to be BHR plasmids, and two of them (pAKD4 and pAKD14) were also captured in TM. We confirmed the ability of the 7 plasmid types captured in TM to mobilize pMOL187 by demonstrating the subsequent transfer of this vector from \textit{R. eutropha} AE815 into \textit{E. coli} CM330. Interestingly, these plasmids conferred mercury resistance to \textit{E. coli} although this phenotype had not been selected for in TM.

### 4. Discussion

Most previous studies done to determine the impact of mercury addition on soil microorganisms have focused on the effect the stress has on community structure and function. These studies have shown that when mercury is present at concentrations in the soil...
range of 1–50 μg HgCl₂ per g soil, it has only occasionally any effect on respiratory activity [39]. We determined the total number of culturable bacteria, and the number of Hg<sup>R</sup> colony forming unit per g of soil and used these data in combination with observed shifts in the abundance of populations (based on differences in colony morphology) to assess changes in community structure that occurred upon addition of mercury. With the exception of the Groningen soil, the addition of 25 μg HgCl₂ per g soil did not cause dramatic changes in total cfu over a 56-day period. However, there was a marked increase in the number of Hg<sup>R</sup> bacteria which was accompanied by an apparent decrease in the diversity of the numerically dominant populations. These data indicate that mercury did exert at least a moderate selective pressure and caused an alteration in the community structure. This is in accordance with the findings of previous colony enumeration studies where comparable mercury concentrations were added to soil [40,41].

A possible role of plasmids in the dissemination of mercury resistance determinants among indigenous soil bacteria was assessed by determining the incidence, diversity and host range of plasmids that confer mercury resistance themselves, as well as those that mediate the mobilization of plasmids by conjugal transfer. This was done by exogenous plasmid isolation using biparental and triparental matings in parallel so that the numbers of donors, recipients and helper cells were comparable in either procedure. This provided a way to directly compare the numbers of transconjugants obtained with each procedure. The results obtained with the two approaches showed that the incidence of both plasmids that confer resistance to mercury and mobilizing plasmids increased in five of the eight soils tested. These findings agree with those of previous investigators who have used biparental mating procedures to show an increase in the number of Hg<sup>R</sup> transconjugants obtained from soils following the addition of mercury to soil [40]. However, to the best of our knowledge, we report for the first time the use of a triparental mating procedure to show a clear increase in the gene mobilization potential of several soils after pollution with a heavy metal.

Interestingly, there was no increase in the frequency of Hg<sup>R</sup> transconjugants obtained with three of the eight soils tested, although the numbers of Hg<sup>R</sup> bacteria increased from <10 to 10<sup>5</sup>–10<sup>7</sup> cfu g<sup>−1</sup> soil. There are several possible explanations for these observations. One is that the genes that confer mercury resistance are predominantly encoded on the chromosome(s) in these Hg<sup>R</sup> bacterial populations, and are not readily transferred by plasmid mediated conjugation. This was suggested by Wickham and Atlas [41], who could not observe any increase in plasmid incidence by endogenous plasmid isolation despite an increase in overall mercury resistance of the isolates. A second possibility is that there were Hg<sup>R</sup> plasmids in these soils, which were not transferable to or stably maintained in the recipient strains used here.

A primary objective of the present study was to assess the incidence of broad-host-range plasmids in bacterial communities adapting to external stress. Of the 140 plasmids obtained from biparental matings, there were 15 types, of which five were found to be broad-host-range plasmids based on their ability to transfer and replicate in bacteria from two different phylogenetic subdivisions. This was also the case for five of the seven different plasmid types captured by triparental matings (the host range of the remaining two plasmids was not determined), wherein the selection imposed was solely on the basis of the ability to mobilize an IncQ plasmid. None of the broad-host-range plasmids obtained hybridized to the rep probes derived from the well known incompatibility groups of broad-host-range plasmids. This is consistent with the findings of several other investigators who have found that broad-host-range plasmids obtained from soil or aquatic environments have novel rep genes that differ from those found in plasmids in clinically significant bacterial species [24–27].

An important question to consider when assessing the potential risk posed by the use of genetically modified microorganisms in environmental biotechnology, is the relative importance of narrow- and broad-host-range plasmids in horizontal gene transfer. In this study we found that narrow-host-range plasmids appeared responsible for most, if not all, of the transfer of Hg<sup>R</sup> genes in 3 of 5 soils. The importance of narrow-host-range plasmids in the dissemination of mercury resistance was also clear from data obtained from the Stends soil. In this soil, two narrow-host-range plasmids were found prior
to mercury addition, and afterwards the plasmid type that was initially predominant (96% of those obtained) was replaced by two other narrow-host-range plasmids, and one broad-host-range plasmid (6%). This suggests that the genetic determinants that confer mercury resistance may often be found on narrow-host-range plasmids. However, these results have to be interpreted with care. *Pseudomonas putida* UWC1 was used as a recipient in these experiments, and it is possible that a different outcome would have been obtained if recipients from other phylogenetic groups were used. The high number of NHR plasmids captured in the *P. putida* recipient could indeed also suggest that the transfer frequency and/or stability of these plasmids in this specific recipient strain is better than for broad-host-range plasmids.

The data obtained from the StendS soil show that not only did the incidence of plasmids increase following the addition of mercury, but so did the diversity of the plasmids obtained. For example, there were two plasmid types recovered prior to mercury addition, whereas four types were recovered afterwards. This is in accord with studies that have reported a higher diversity of plasmids in polluted environments than in non-polluted sites [42]. However, such data should be cautiously interpreted since the apparent increased diversity of plasmids may not simply be the outgrowth of preexisting populations. Many *mer* determinants are located on transposons [1] that have been shown to readily transfer from a chromosome to conjugative plasmids [43,44]. Consequently, the observed increase in plasmids that confer mercury resistance could simply be due to transposition of the *mer* genes from one plasmid to another. Further studies on the diversity of *mer* genes encoded on the various plasmids are needed to distinguish between these possibilities.

All of the plasmids isolated using biparental matings were tested for their ability to mobilize the IncQ vector pMOL187 into *R. eutropha*. In addition, all the plasmids obtained in triparental matings were tested to determine if they conferred resistance to mercury in *E. coli*. Thus, all the plasmids were tested using the isolation criteria of the opposite procedure. None of the 10 narrow-host-range plasmids, but 4 of the 5 broad-host-range plasmid types, captured using biparental mating were able to mobilize the IncQ vector pMOL187 into *R. eutropha*. Similar results were obtained in a separate study using the same soils (Drönen et al. [45]) wherein 9 of 11 broad-host-range plasmids (that were different from those obtained in this study) could mobilize the IncQ vector. These observations suggest that IncQ mobilization is a property often associated with broad-host-range plasmids. All of the 7 broad-host-range plasmids captured in triparental matings were found to encode mercury resistance. This was not necessarily expected since this method did not select for Hg$^{R}$ plasmids. It indicates that the addition of mercury to the soil did not enhance the abundance of broad-host-range plasmids in general, but especially increased those which conferred resistance to mercury.

Of the 7 different Hg$^{R}$ plasmid types obtained using triparental matings, 5 were not found among the 140 transconjugants examined from biparental matings. Of these 5, two plasmids were, however, able to transfer to *P. putida*, as shown in plate matings (Table 6). There could be several reasons why they were not captured in *P. putida* during the biparental exogenous isolation with soil. First, the number of strains that harbored these BHR plasmids in the soils could have been much lower than the number of cells with Hg$^{R}$ NHR plasmids. Secondly, the transfer frequency or stability in *P. putida* could be lower for these BHR plasmids than for the Hg$^{R}$ NHR plasmids found in this recipient. However, 2 of the 7 plasmids were found with both procedures, based on their identical EcoRI digest patterns. One of these, pAKD4, was captured with both methods from two soils that were located 400 m apart (StendO and StendS).

The results of this study show that the use of biparental and triparental mating procedures provides a more complete understanding of the incidence and diversity of plasmids in soils and their contribution to gene flow in natural communities than one method alone. This might be extended even further if the hosts used in a given mating procedure were drawn from various phylogenetic groups such as the α and γ divisions of the Proteobacteria.

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