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Mercury resistance determined by a self-transmissible plasmid in *Bacillus cereus* 5

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Summary. Inducible mercuric reductase activity in *Bacillus cereus* 5 was plasmid-encoded. Plasmid analysis revealed three plasmids with molecular masses of 2.6, 5.2 and 130 MDa. A mating system permitted transfer of the resistance determinant among strains of *B. cereus* and *B. thuringiensis*. Transfer of mercury resistance from *B. cereus* 5 to *B. cereus* 569 and *B. thuringiensis* occurred during mixed culture incubation on agar surfaces. The 130-MDa plasmid (pGB130) was responsible for transfer; frequencies ranged from 10^{-5} to 10^{-4}. *B. cereus* 569 transconjugants inheriting pGB130 were also effective donors. High transfer frequencies and the finding that cell-free filtrates of donor cultures were ineffective in mediating transfer suggested mercury-resistance transfer was not phage-mediated. Transfer was also insensitive to DNase activity. Further evidence that pGB130 DNA carried the mercury-resistance determinant was transformation of *B. cereus* 569 by electroporation with pGB130 DNA isolated from *B. cereus* 5 and a mercury-resistant *B. cereus* 569 transconjugant. Mercury-resistant transconjugants and transformants exhibited mercuric reductase activity. Plasmid pGB130 also conferred resistance to phenylmercuric acetate.

Key words: Mercury – Plasmid – *Bacillus cereus* – Conjugation – Electroporation

Introduction

Mercury resistance (Dyke et al. 1970; Richmond and John 1964), enzymatic reduction of mercuric mercury (Hg^{2+}) to volatile elemental mercury (Hg^0), and decomposition of organomercurial compounds by mercury-resistant bacteria has been demonstrated in Gram-negative and Gram-positive bacteria (Barkay et al. 1985; Clark et al. 1977; Furukawa et al. 1969; Izaki 1981; Jobling et al. 1988). Two distinct mercury-resistance phenotypes involving enzymatic transformation and volatilization of Hg^0 from inorganic mercury and organomercurial substrates have been described; (a) detoxification of organomercurial compounds by broad-spectrum resistance determinants is a two-step process that includes cleavage of the C-Hg bond by an intracellular organomercurial lyase enzyme followed by reduction of released Hg^{2+} to Hg^0 by the FAD-containing, NADPH-dependent enzyme, mercuric reductase (Schottel 1978; Tezuka and Tonomura 1978); (b) narrow-spectrum, mercury-resistance determinants confer resistance only to inorganic mercury (i.e. Hg^{2+}) via mercuric reductase. Reductive mercury resistance is inducible by subinhibitory concentrations of Hg^{2+} or organomercurials (Brown 1985).

Widespread distribution of mercury resistance is partly due to determinants on plasmids or transposons, particularly in Gram-negative bacteria (Foster 1987). However, *mer* determinants are also located on the chromosome of Gram-positive *Staphylococcus aureus* and *Bacillus* species (Wang et al. 1987; Witte et al. 1986). When the present study commenced, the only Gram-positive species, in addition to strains of *S. aureus*, that was shown to possess a mercuric reductase enzyme system was *Bacillus cereus* 5 (Izaki 1981). This strain is resistant to Hg^{2+}, produces volatile Hg^0 from both Hg^{2+} and Hg^+ and was reported to be sensitive to phenylmercuric acetate. It was concluded that *B. cereus* 5 possessed a narrow-spectrum *mer* determinant but no information was available on the plasmid-encoded or chromosomal nature of resistance. Timoney et al. (1978) reported a marine *Bacillus* species volatilized Hg^{2+} to Hg^0 but no evidence as to the location of the resistance determinants or follow-up reports have been published. Mercuric reductase activity has been demonstrated in other Gram-positive bacteria including *Mycobacterium scrofulaceum* (Meissner and Falkinham 1984). *Clostridium perfringens* (Rudrik et al. 1985), *Streptococcus agalactiae* and *Streptomyces lividans* (Nakahara et al. 1985). Mahler et al. (1986) described broad-spectrum mercury resistance in *Bacillus* strain

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RC607; this was shown by Southern blot analysis with the *S. aureus* broad-spectrum *mer* determinant of plasmid pI258 to be chromosomally encoded (Wang et al. 1987; Wang et al. 1989).

Efforts to locate the *mer* determinant in *B. cereus* 5 by colony hybridization with a *mer* DNA probe from the *Escherichia coli* narrow-spectrum determinant of plasmid R100 (Barkay 1985) and from *Bacillus* sp. strain RC607 (Wang et al. 1987) have been unsuccessful, indicating that the *mer* nucleotide sequence in *B. cereus* 5 is not homologous with those of known Gram-negative and Gram-positive systems. Since DNA–DNA homology studies were unsuccessful in locating the *mer* determinant, the present study investigated the resistance determinant in *B. cereus* 5 using conjugation and transformation (by electroporation) of the presumed *mer* determinant to mercury-sensitive recipients.

Materials and methods

Bacterial strains and plasmids. Bacillus cereus, B. thuringiensis, Escherichia coli and *Agrobacterium tumefaciens* strains and plasmids are listed in Table 1. Transcipient strains from mating and transformation experiments are identified by the abbrevation tc for transconjugants, or tf for transformants. In the mating system, the first term (e.g. 569 UM20-1 in the label 569 UM20-l [pGB130] Hg^r-tc 104) designates the recipient from which the transconjugant was derived, and the second term (e.g. [pGB130] Hg^r-tc 104) identifies a presumptive Hg^r transconjugant purified by single-colony isolation. Transconjugants from mating mixtures in which *B. cereus* 5 was the donor are primary transconjugants. Secondary transconjugants were from matings in which donors were Hg^r *B. cereus* 569 primary transconjugants containing pGB130.

Media and growth conditions. Growth media included Luria-Bertani (LB) broth (Acumedia Manufacturers, Inc., Baltimore, MD or Difco Laboratories, Detroit, MI) and Difco Brain Heart Infusion (BHI) broth. For solid media, 15 g agar/l broth was added. Peptone diluent was prepared by adding 1 g peptone/l deionized water. When necessary, media were supplemented with chloramphenicol (5, 10, 15 or 20 μg/ml), streptomycin (50 or 200 μg/ml), or tetracycline (10 or 25 μg/ml). For selection of mercury-resistant (Hg^r) cells, $HgCl_2$ (10, 25, 35 or 50 μM) was used.

Plasmids and chromosomal DNA isolation. Numerous plasmid isolation methods and modifications (Anderson and Mackay 1983; Bernhard et al. 1978; Bingham et al. 1979; Birnboim and Doly 1979; Gonzalez et al. 1981; Kado and Liu 1981) were initially used without success. Numerous parameters were modified including stage of cell growth, i.e. logarithmic versus stationary phase; predigestion of cells with lysozyme treatment; and different concentrations of lysozyme. Additional variables included: (a) lysis of cells wtih Triton X-100 or sodium dodecyl sulfate (SDS); (b) heat shock, alkali treatment of lysozyme and detergent-treated cells; (c) deproteination of lysates with phenol/chloroform and/or chloroform/isoamyl alcohol; (d) and precipitation of DNA with ethanol or isopropanol. The vertical in-well-lysis technique described by Gonzalez et al. (1981) allowed detection of the 2.6-MDa and 5.2-MDa plasmids in *B. cereus* 5. Battisti et al. (1985) described an alkaline-SDS method for isolating plasmids from *B. thuringiensis, B. cereus* and *B. anthracis* strains. This technique was used to isolate the 2.6-MDa and 5.2-MDa plasmids from *B. cereus* 5 (data not presented). An NaCl precipitation treatment and an additional phenol extraction of cell lysates optimized plasmid yields from *B. cereus* 5. Reference plasmids from *B. thuringiensis* 4042A UM8td2 were always detectable. A small-scale version of this technique was developed. No change was noted in the plasmid content of *B. thuringiensis* 4042A UM8td2, but an additional 130-MDa plasmid was detected in *B. cereus* 5. Extraction of plasmid DNA was therefore carried out using the small-scale alkaline-SDS method (Belliveau and Trevors 1989). Chromosomal DNA was isolated by the method of Wilson and Morgan (1984).

Determination of plasmid molecular mass and conformation. Molecular masses of plasmids in *B. cereus* 5 were estimated from electrophoretic mobilities of reference plasmids in *E. coli* V517, *B. thuringiensis* subsp. *thuringiensis* 4042A UM8dt2 and *A. tumefaciens* ID135. Heat denaturation (Jarrett 1983) was used to determine the conformation of plasmids in *B. cereus* 5. A 10-μl sample

Table 1. Bacterial strains and plasmids

Strain	Relevant characteristics	Relevant plasmid(s) and/or molecular mass (MDa)	Origin or reference
Agrobacterium tumefacienes ID135	reference plasmids	260, 130	S. Meissner[a]
Bacillus cereus 5	Hg^r	130, 5.2, 2.6	K. Izaki[b]
B. cereus 569 UM20-1	Ant^-, Str^r	12	C. Thorne[c]
B. cereus 569 UM20-1 Cm^r-tf1	Ant^-, Str^r, Cm^r	pC194	This laboratory
B. thuringiensis susp. *thuringiensis* 4042A UM8td2	Ade^-, Cry^+, Tc^r	pX011, pX012, pBC16	C. Thorne[c]
Escherichia coli V517[d]	reference plasmids	35.8, 4.8, 3.7, 3.4 2.6, 2.0, 1.4	Marcina et al. (1978)

Abbreviations: Hg^r, mercury-resistant; Str^r, streptomycin-resistant; Ant^-, anthranilic acid requirement; Cm^r, pCl94-encoded chloramphenicol resistance; Ade^-, adenine requirement; Cry^+, pX012-encoded entomocidal parasporal crystal toxin production; Tc^r, pBC16-encoded tetracycline resistance

[a] Virginia Polytechnic Institute and State University, Blackburg, Virginia USA

[b] Tohoku University, Sendai, Japan

[c] University of Massachusetts, Amherst, Massachusetts, USA

[d] Strain is missing the 1.8-MDa plasmid described in cited reference

of plasmid DNA was heated at 100° C for 5 min, cooled on ice and subjected to agarose gel electrophoresis. Denaturated DNA did not enter the agarose gel under the electrophoresis conditions used (i.e. 7 V/cm, 2 h, 0.7% mass/vol. agarose).

Mating conditions. Donor and recipient cultures were individually grown in 10 ml BHI broth supplemented with either 25 μM $HgCl_2$, 200 μg/ml streptomycin, 20 μg/ml chloramphenicol or 25 μg/ml tetracycline for selection of either *B. cereus* 5, *B. cereus* 569 UM20-1, *B. cereus* 569 UM20-1 (pC194) Cm^r-tfl or *B. thuringiensis* 4042A UM8td2, respectively. Donor and recipient strains were subcultured after 14-h growth into 10 ml BHI broth containing either $HgCl_2$ (10 μM), streptomycin (50 μg/ml), chloramphenicol (5 μg/ml) or tetracycline (10 μg/ml), as appropriate and incubated for 10 h at 30° C with shaking at 100 rpm. Late-log-phase donor and recipient strains, each containing approximately 5×10^7 colony forming units (cfu)/ml were centrifuged at $4000 \times g$ for 10 min (20° C) and resuspended in an equal volume of sterile BHI broth. Equal volumes (0.5 ml) of donor and recipient cells were mixed, and a 40-μl aliquot (10^6–10^7 cells) of each organism was spread over a 5 cm^2 area on BHI agar and incubated for 24 h at 30° C. Bacterial growth was removed with a sterile spatula, suspended in 1 ml 0.1% (mass/vol.) peptone diluent, and plated on selective LB agar to determine number of donors, recipients, and transconjugants. Plates were incubated at 30° C and cfu enumerated after 24–48 h. When mating mixtures were prepared with streptomycin-resistant (Str^r) recipients and mercury-resistant (Hg^r) donors, mercury-resistant transconjugants were selected on LB agar supplemented with 200 μg/ml streptomycin and 35 μM $HgCl_2$. In mating mixtures with tetracycline-resistant (Tc^r) recipients and mercury-resistant donors, mercury-resistant transconjugants were selected on LB agar supplemented with 10 μg/ml tetracycline and 35 μM $HgCl_2$. In matings with chloramphenicol-resistant (Cm^r) recipients and mercury-resistant donors, mercury-resistant transconjugants were selected on LB agar containing both 15 μg/ml chloramphenicol and 35 μM $HgCl_2$. Transfer frequencies were expressed as transconjugant cfu/ml divided by donor cfu/ml. To determine mutation frequency to mercury and antibiotic resistance, donor and recipient strains were plated separately on LB agar. To ensure that double selection agar did inhibit the growth of donors and recipients, separate cultures of each were plated on this medium.

Effect of DNase, donor filtrates and separation of donor and recipient cells on transfer of mercury resistance. Cells resuspended in BHI broth (supplemented with 5 mM $MgCl_2$) containing 1 mg/ml DNaseI were spotted on BHI agar containing 1 mg/ml DNase. BHI broth supplemented with $MgCl_2$, but without DNase was added to control mixtures. DNase activity under these conditions was tested in controls consisting of *B. cereus* 5 genomic DNA: 20-μg samples of DNA were spread on sterile 0.45-μm cellulose nitrate filters on BHI agar containing no DNase and on BHI agar containing DNase. To test DNA-degrading activity, BHI agar was supplemented with DNase during mating. DNA was spread on separate filters on appropriate BHI agar plates after 0, 4, 10, 12 and 22 h from the initial time of incubation and samples incubated at 30° C for 24 h. Filters were removed, placed in sterile tubes and the DNA suspended in 1 ml (10 mM Tris, 1 mM Na_2 EDTA, pH 7.0). DNA was precipitated with 0.1 vol. 3 M sodium acetate and 2 vol. absolute ethanol for 18 h at −20° C, and electrophoresed as described previously (Belliveau and Trevors 1989). To investigate the possibility of phage-mediated plasmid transfer, cell-free filtrates of donor cultures were substituted for donor cells. The supernatant fluid from 1 ml donor culture was filtered through a sterile 0.45-μm cellulose nitrate filter and 0.2 ml of the cell-free filtrate mixed with 20 μl recipient cell suspension, then spot-plated onto BHI agar. Mixtures were incubated and observed for mercury-resistant transcipients. To demonstrate that cell-to-cell contact was necessary for DNA transfer, a 20-μl aliquot of donor cell culture was applied to a sterile 0.45-μm cellulose nitrate filter placed on a fresh lawn of the recipient strain on BHI agar so as to separate the two cultures by the filter. After incubation for 24 h at 30° C the filter was removed and donor cells suspended in 1 ml peptone diluent. Recipient cell growth under the filter was removed and Hg^r transcipients enumerated by plating on selective LB agar. Colonies were counted after a 24–48-h incubation.

Transformation by electroporation. The Gene Pulser electroporation apparatus (Bio-Rad, Mississauga, Canada) was used to transform *B. cereus* 569 UM204 with plasmid DNA from *B. cereus* 5 and *B. cereus* 569 UM20-1 (pGB130) Hg^r-tc104, a mercury-resistant transconjugant isolated from the mating between *B. cereus* 5 and the recipient *B. cereus* 569 UM20-1 strain. Recipient cells were grown to late-log phase (2×10^8 cfu/ml) in LB broth, washed three times and suspended in 4 ml electroporation buffer (10 mM HEPES pH 7.0) to yield 5.5×10^8 cfu/ml. A 0.8-ml aliquot of cells and 0.5 μg pGB130 were mixed and electroporated under optimum conditions (capacitance 3 μF, voltage 1.5 kV, field strength 3750 V/cm; Belliveau and Trevors 1989). Transformants were selected on LB agar containing 25 μM $HgCl_2$. The frequency of transformation was expressed as the number of transformant cfu/ml divided by total recipient cfu/ml before electroporation. Plasmid transfer efficiency was expressed as transformant cfu/ml divided by mass (μg) of pGB130 DNA. To calculate percentage survival of *B. cereus* 569 UM20-1 after electroporation, the initial cfu/ml before electroporation and cfu/ml recovered after electroporation were determined by serial dilution and plating on LB agar.

Resistance of bacterial strains to $HgCl_2$ and phenylmercuric acetate. Presumptive mercury-resistant transconjugants and transformants were tested for sensitivity to $HgCl_2$ and phenylmercuric acetate (PMA) by the disc diffusion assay (Weiss et al. 1977). Cultures were grown in 5 ml BHI broth at 30° C with shaking until they reached an absorbance at 600 nm of 1.0. A 0.1-ml sample of each strain was spread onto a separate BHI agar plate and sterile 6.5-mm-diameter cellulose discs, loaded with either $HgCl_2$ (at 10, 25, 50, 100 and 200 nmol/disc) or PMA (at 2.5, 5, 10 and 20 nmol/disc), were aseptically placed on the agar surface. Inhibition of bacterial growth was measured after a 16-h incubation at 30° C, and recorded as diameter of inhibition minus diameter of disc. The mercury-resistant *B. cereus* 5, and mercury-sensitive *B. cereus* 569 UM20-1 and *B. thuringiensis* 4042A UM8td2, were used as controls. Strains with an inhibition zone of 7–17 mm were supersensitive (Hg^{ss}), sensitive strains (Hg^s) had a zone of inhibition 2–7 mm, and Hg^r strains had a zone of inhibition less than 0.5 mm. Sensitivity of strains to PMA was generally 10-fold greater than to $HgCl_2$; strains with a zone of inhibition 15–21 mm in the presence of PMA were supersensitive (PMA^{ss}) while sensitive strains (PMA^s) had an inhibition zone 7–15 mm and resistant strains (PMA^r) less than 5 mm.

Reduction of Hg^{2+} by mercuric reductase enzyme activity. $HgCl_2$-dependent oxidation of NADPH was determined by the decrease in absorbance at 340 nm with cell-free extracts from Hg^r strains using methods reported previously (Izaki 1981; Rinderle et al. 1983). Each culture was grown for 8–12 h in BHI broth supplemented with subinhibitory concentrations of $HgCl_2$ (25 μM) or PMA (5 or 10 μM). Hg^r and Hg^s strains were grown in the absence of either $HgCl_2$ or PMA. A 2-ml sample of each culture was centrifuged for 5 min at 4° C in an Eppendorf centrifuge. Cells were washed twice and suspended in 1 ml 50 mM phosphate pH 7.0, cooled on ice and disrupted by sonication. Cell-free extracts were obtained by centrifugation at $15600 \times g$ for 5 min at 4° C. Assay mixtures contained 0.5 ml cell-free extract, 50 mM phosphate pH 7.0, 100 μM $HgCl_2$, 100 μM NADPH, 0.5 mM Na_2 EDTA, and 1 mM 2-mercaptoethanol in a volume of 3 ml. One unit of activity is the amount of enzyme causing an initial decrease in A_{340} at 30° C of 0.01 min^{-1}; specific activity is defined as units/mass protein. Protein concentrations were determined by the method of Bradford (1976), with Bio-Rad protein dye reagent, and bovine serum albumin as the standard.

Results

Transfer of $HgCl_2$ resistance

To study the involvement of plasmids in *B. cereus* 5 (Fig. 1, lane 2) with mercury resistance, we investigated whether the *B. thuringiensis* mating system (Battisti et al. 1985) could be applied to *B. cereus* 5. Two large plasmids (pX011 and pX012) in *B. thuringiensis* 4042A UM8td2, were shown by Battisti et al. (1985) to be self-transmissible, each being capable of promoting its own transfer, as well as that of other plasmids such as the tetracycline-resistance plasmid pBC16, to strains of *B. cereus* and *B. anthracis*. The strategy for using *B. cereus* 5 as a recipient in matings with *B. thuringiensis* 4042A UM8td2 was to transfer either or both fertility plasmids to *B. cereus* 5 and subsequently mobilize plasmid(s) and $HgCl_2$ resistance from *B. cereus* 5 to a Hg^s recipient like *B. cereus* 569 (Fig. 1).

In preliminary plate matings with *B. thuringiensis* 4042A UM8td2 and *B. cereus* 5, Hg^rTc^r transconjugants were detected at a frequency of 1.1×10^{-5} (Table 2). Examination of donor and recipient strains, as well as 20 Hg^r,Tc^r transconjugants, revealed recipients were *B. thuringiensis* 4042A UM8td2 and not *B. cereus* 5. This indicated the direction of transfer was from *B. cereus* 5 to *B. thuringiensis* 4042A UM8td2, opposite to the expected direction. Plasmid analysis by agarose gel electrophoresis revealed the Hg^r transconjugants had not acquired any plasmids from *B. cereus* 5. This initial evidence suggested that mercury resistance in *B. cereus* 5 was not linked to any plasmids and was assumed to be chromosomally encoded.

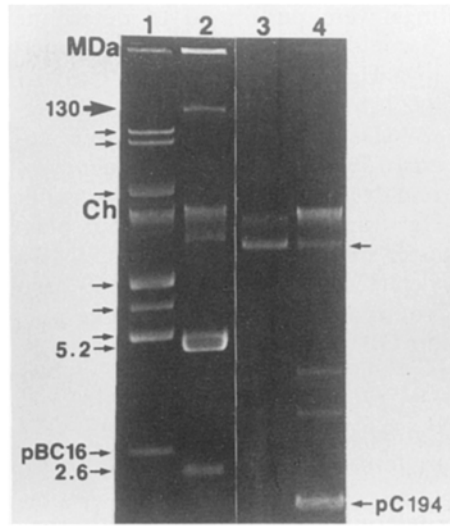

Fig. 1. Agarose gel electrophoresis of plasmids in *B. cereus* and *B. thuringiensis* subsp. *thuringiensis* strains. Ch, indicates of chromosomal DNA; arrows indicate positions of plasmid DNA. Plasmid designations or estimated molecular masses are listed in brackets with strain designations. Lane 1, *B. thuringiensis* subsp. *thuringiensis* 4042A UM8td2, recipient (pX012 [top], pX011, ≈27, ≈10, ≈8 and ≈6 MDa, and pBC16 [2.8 MDa]; lane 2, *B. cereus* 5, Hg^r donor (130, 5.2, and 2.6 MDa); lane 3, *B. cereus* 569 UM20-1, recipient (≈12 MDa); lane 4, *B. cereus* 569 UM20-1 Cm^rtfl, recipient (≈12 MDa, and pCl94 [1.8 MDa])

Table 2. *B. cereus* and *B. thuringiensis* as recipients, and effectiveness of transconjugants as donors of pGB130 and Hg^r phenotype

Mating (donor × recipient)	Selection of transcon-jugants	Plasmid transferred	Hg^r transcon-jugant (cfu/ml)	Transfer frequency
Primary transconjugant				
B. cereus 5 × *B. cereus* 569 UM20-1	Hg^r Str^r	pGB130	4.2×10^4	3.5×10^{-4}
B. cereus 5 × *B. cereus* 569 UM20-1 (pC194) Cm^r-tfl	Hg^r Cm^r	pGB130	8.3×10^2	2.8×10^{-5}
B. cereus 5 × *B. thuringiensis* 4042A UM8td2	Hg^r Tc^r	None[a]	2.0×10^2	1.1×10^{-5}
Secondary transconjugant				
B. cereus 569 UM20-1 (pGB130) Hg^r-$tc104_x$ *B. cereus* 569 UM20-1 (pCl94) Cm^r-tfl	Hg^r Cm^r	pGB130	9.6×10^2	3.4×10^{-5}
B. thuringiensis 4042 A UMtd2 Hg^r-tc212 × *B. cereus* 569 UM20-1	Hg^r Str^r	0	0	0

In strains designations, tf in the second term denotes chloramphenicol-resistant transformant obtained by electroporation with plasmid pCl94; tc denotes presumed Hg^r transconjugant of plasmid pGB130. All values are averages of results from at least two experiments

[a] pGB130 was not detected in transconjugants

Additonal matings were performed to determine whether *B. cereus* 5 could transfer the mercury-resistance determinant to a Hg^sStr^r *B. cereus* 569 UM20-1 recipient. The results (Table 2) showed that mercury resistance and the 130-MDa plasmid (pGB130) were co-transferred to *B. cereus* 569 UM20-1 at a frequency of 3.5×10^{-4}. Plasmid analysis (Fig. 2) confirmed transfer of pGB130 to all Hg^r transconjugants tested for plasmid DNA. Co-transfer of the 2.6-MDa and 5.2-MDa plasmids was not detected in any *B. cereus* 569 recipients which had acquired the mercury-resistance phenotype and plasmid pGB130.

Transfer of pGB130 from B. cereus *569 and* B. thuringiensis *primary transconjugants*

It was necessary to determine whether the primary Hg^r-resistant transconjugants from *B. cereus* 569 UM20-1 matings could act as donors of pGB130 and the mercury-resistance phenotype. Since *B. cereus* 569 UM20-1 was an effective recipient of pGB130 from *B. cereus* 5, the former organism was chosen as the recipient in matings with a primary Hg^r transconjugant. To serve as a recipient in this mating, *B. cereus* 569 UM20-1 was given a selectable phenotype (other than Hg^r) different from the primary transconjugant. Therefore, *B. cereus* 569 UM20-1 was transformed by electroporation with pCl94, a chloramphenicol-resistance (Cm^r) plasmid (Belliveau and Trevors 1989).

The resultant mating was as follows: *B. cereus* 569 UM20-1 (pGB130) Hg^r-tc104 primary transconjugant (donor) × *B. cereus* 569 UM20-1 (pCl94) Cm^r-tfl transformant (recipient). Secondary transconjugants were designated as *B. cereus* 569 UM20-1 (pCl94, pGB130) Cm^rHg^r-tc. The Hg^r primary transconjugant (*B. cereus* 569 UM20-1 [pGB130]) was an effective donor of pGB130 to *B. cereus* 569 UM20-1 (pCl94) Cm^r-tfl (Fig. 2) with a plasmid transfer frequency of 3.4×10^{-5} (Table 2).

The effect of pCl94 on transfer of pGB130 was determined by performing the mating: *B. cereus* 5 donor × *B. cereus* 569 UM20-1 (pCl94) Cm^r-tfl recipient. The transfer frequency of pGB130 was 2.8×10^{-5} (Table 2). The presence of pCl94 in the recipient resulted in a 10-fold decrease in transfer frequency of pGB130, since the original mating of *B. cereus* 5 × *B. cereus* 569 UM20-1 displayed a transfer frequency of 3.5×10^{-4} (Table 2). However, since the primary transconjugant *B. cereus* 569 UM20-1 (pGB130) Hg^r-tc104 transferred pGB130 at a frequency of 3.4×10^{-5}, comparable to the transfer frequency of pBG130 from *B. cereus* 5 (2.8×10^{-5}), the primary transconjugant was considered as effective a donor of pGB130 as *B. cereus* 5. Agarose gel electrophoresis of plasmid DNA provided physical evidence of pGB130 transfer. A selected *B. thuringiensis* 4042A UM8td2 primary Hg^r transconjugant (UM8td2 Hg^r-tc212) did not function as a donor of mercury resistance to *B. cereus* 569 UM20-1 using plate mating conditions (Table 2).

Mechanism of pGB130 transfer

To determine if transfer of plasmid DNA occurred by transformation during matings, the sensitivity of pGB130 transfer to DNase activity was examined (data not shown in tables). In matings between *B. cereus* 5

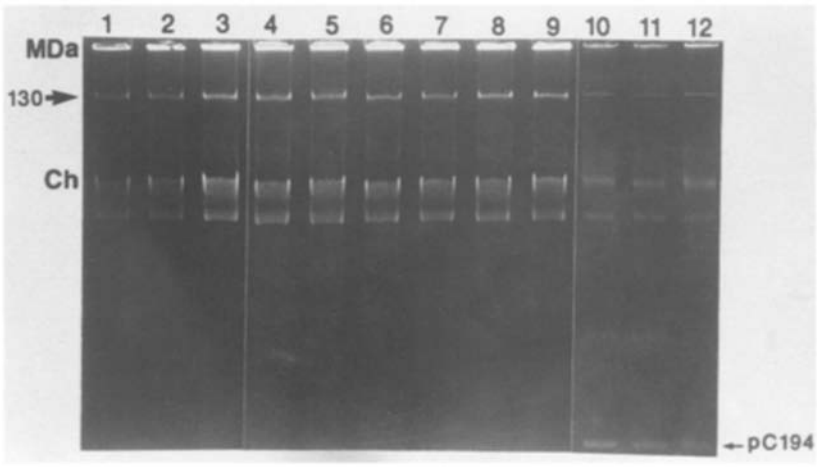

Fig. 2. Agarose gel electrophoresis of plasmids in *B. cereus* primary and secondary transconjugants and transformants. Plasmid designations are the same as in Fig. 1. Lane 1, *B. cereus* 569 UM20-1 Hg^r-tc103, primary transconjugant; lane 2, *B. cereus* 569 UM20-1 Hg^r-tc104, primary transconjugant; lane 3, *B. cereus* 569 UM20-1 Hg^r-tc111, primary transconjugant; lane 4, *B. cereus* 569 UM20-1 Hg^r-tf20, primary transformant; lane 5, *B. cereus* 569 UM-20-1 Hg^r-tf32, primary transformant; lane 6, *B. cereus* 569 UM20-1 Hg^r-tf63, primary transformant; lane 7, *B. cereus* 569 UM20-1 Hg^r-tf2, secondary transformant; lane 8, *B. cereus* 569 UM20-1 Hg^r-tf4, secondary transformant; lane 9, *B. cereus* 569 UM20-1 Hg^r-tf17, secondary transformant; lane 10, *B. cereus* 569 UM20-1 Cm^rHg^r-tc306, secondary transconjugant; lane 11, *B. cereus* 569 UM20-1 Cm^rHg^r-tc307, secondary transconjugant; lane 12, *B. cereus* 569 UM20-1 Cm^rHg^r-tc308, secondary transconjugant

donor and *B. cereus* 569 UM20-1 recipient strains, the number of Hg^r transconjugants (5.7×10^{-4}) detected after 24 h in the presence of DNase and $MgCl_2$ was not appreciably different from the number obtained in presence of $MgCl_2$ alone (2.1×10^{-4}). In matings between *B. cereus* 5 donor and *B. thuringiensis* 4042A UM8td2, the number of Hg^r transconjugants obtained in the presence of DNase plus $MgCl_2$ (1.0×10^{-5}) was also not substantially different from the number obtained in the presence of $MgCl_2$ alone (1.1×10^{-5}). Agarose gel electrophoresis of controls containing *B. cereus* 5 DNA revealed the DNA was completely degraded by DNase, while no digestion occurred in BHI gar without DNase (figure not shown).

To investigate the possibility of phage-mediated plasmid transfer, cell-free filtrates prepared from donor cultures were tested for their ability to transduce recipients to mercury resistance. No Hg^r transcipients could be detected, suggesting that pGB130 was not transferred to recipients by transductions. No Hg^r recipient colonies were isolated when donor and recipient cells were physically separated by a 0.45-μm membrane filter, suggesting that the mechanism of transfer was not transformation and cell-to-cell contact was required.

Plate matings of donor *B. cereus* 5 and recipient *B. cereus* 569 UM20-1 directly on BHI agar containing both mercury (35 μM $HgCl_2$) and streptomycin (200 μg/ml) did not produce Hg^r transconjugants (data not presented). Colony growth that occurred after a 24-h incubation under double selection consisted of recipient *B. cereus* 569 UM20-1 cells alone. Gratuitous mercury resistance-like growth was possible if a heavy inoculum of *B. cereus* UM20-1 cells was spread onto BHI agar containing normally inhibitory concentrations of mercury (25–50 μM $HgCl_2$). Therefore, the conjugation-like process between donor and recipient strains probably occurred during 24 h of mixed-culture incubation on non-selective BHI agar rather than after selection for transconjugants on selective LB agar. No spontaneous mutants of either donor or recipient strains were isolated from mating controls on agar under double selection. Plasmid transfer was not obtained when donor and recipient cells were grown together in BHI broth (data not presented). Cell-to-cell contact is believed to be necessary for the transfer process to occur, similar to findings reported by Fischer et al. (1984).

Transformation of B. cereus *569 UM20-1 by electroporation*

Electroporation was used to transform *B. cereus* 569 UM20-1 with *B. cereus* 5 plasmid DNA, which contained a mixture of pGB130 and the cryptic 2.6-MDa and 5.2-MDa plasmids, and plasmid DNA isolated from *B. cereus* 569 UM20-1 (pGB130) Hg^r-tc104 (containing pGB130 and the native cryptic 12-MDa plasmid). In the system used for labelling transformants, the first term (for example 569 UM20-1 in the label 569 UM20-1 [pGB130] Hg^r-tf20) designates the recipient from which the transformant was derived, and the second term (e.g. [pGB130] Hg^r-tf20) identifies a particular Hg^r transformant. Plasmid mixtures from *B. cereus* 5 and the Hg^r transconjugant UM20-1 (pGB130) Hg^r-tc104 transformed *B. cereus* 569 UM20-1 to mercury-resistance phenotype (Table 3). Estimated transformation frequencies (2×10^{-5}) and efficiencies (1×10^4/μgDNA) were slightly lower than transformation by pC194 DNA but in the range considered moderate to high for transforming *Bacillus* species compared to conventional methods based on transformation of competent cells or protoplasts. At least 20 presumptive Hg^r transformants from each electroporation experiment were tested for plasmid DNA by agarose gel electrophoresis of cleared lysates. All transformants had acquired pGB130 and neither of the two smaller cryptic plasmids from *B. cereus* 5 were detected in any isolates. Fig. 2 shows plasmid profiles of selected Hg^r transformants from both transforming DNA mixtures. This evidence strongly

Table 3. Transformation of *B. cereus* 569 UM20-1 by electroporation with plasmid DNA from *B. cereus* 5 and *B. cereus* 569 UM20-1 (pGB130) Hg^r-tc104

Source of plasmid	Plasmid DNA mixture	Field strength (V/cm)	Capacitance (μF)	Time constant (ms)	Survival (%)	Plasmids detected (Hg selection)	Frequency (cfu_{obs}/cfu_{total})	Efficiency (cfu_{obs}/μg pGB130)
B. cereus	pBG130, 5.2-MDa, 2.6-MDa	3750	3	1.2	95.6	pGB130	1.0×10^{-5}	9.5×10^3
B. cereus 569, UM20-1 (pGB130) Hg^r-tc104	pGB130, 12-MDa	3750	3	1.3	94.3	pGB130	4.3×10^{-6}	4.0×10^3

The cryptic 2.6-MDa and 5.2-MDA plasmids from *B. cereus* 5 were not detected in 20 transformants tested for plasmid DNA. Survival was measured as cfu/ml recovered after electroporation as a percentage of the total used in the experiment. cfu_{obs} = colony forming units/ml on LB agar plates containing 25 μM $HgCl_2$; equals cells transformed with pGB130. Values are averages of results from two experiments

suggested that the inorganic-mercury-resistance determinant in *B. cereus* 5 resides on plasmid pGB130.

Susceptibility of Hg^r transconjugants and transformants to $HgCl_2$ and PMA

The distinction between resistance and sensitivity was easily determined for $HgCl_2$ and phenylmercuric acetate (PMA) at concentrations ranging over 10–200 nmol $HgCl_2$/disc and 2.5–20 nmol PMA/disc. Hg^r *B. cereus* 569 UM20-1 transconjugants and transformants, and *B. thuringiensis* 4042A UM8td2 primary transconjugants were more resistant to both mercurials than original recipients. All Hg^r transconjugants and transformants selected for the assay were as resistant to $HgCl_2$ and PMA as *B. cereus* 5. PMA was more toxic to *B. cereus* and *B. thuringiensis* strains than inorganic Hg^{2+}. It was previously reported (Izaki 1981) that *B. cereus* 5 was sensitive to PMA, but it is apparent from this study that *B. cereus* 5 is resistant to PMA, and resistance is linked to the presence of pGB130.

Mercuric reductase activity

To verify mercuric reductase activity, presumably encoded by pGB130, was responsible for Hg^{2+} resistance in transconjugants and transformants of *B. cereus* 569 UM20-1 and *B. thuringiensis* 4042A UM8td2, mercuric reductase enzyme activity was assayed in cell-free extracts. All Hg^r PMA^r strains exhibited mercuric reductase activities between 82.5–121% of *B. cereus* 5 (11 U/mg protein) when cells were induced with $HgCl_2$ (data not shown). All Hg^r PMA^r transconjugants and transformants tested also exhibited mercuric reductase activity when induced with PMA. Enzyme activities were similar to activities expressed when cells were induced with $HgCl_2$ (data not presented). Enzyme activity varied over 81%–143% of the level expressed by *B. cereus* 5 (10.8 U/mg protein). The fact that PMA-induced cells expressed high levels of mercuric reductase activity strongly suggested that pGB130 may confer resistance to organomercurials by organomercurial lyase activity. The purpose of the mercuric reductase assays was to verify the presence of the enzyme in Hg^r recipients, not to compare expression levels.

In the present study, *B. cereus* 5 and all Hg^r PMA^r transconjugants and transformants were equally resistant to PMA, and PMA appeared to induce mercuric reductase activity equally as well as $HgCl_2$. When these same strains were not induced with either $HgCl_2$ or PMA their mercuric reductase activities were negligible, with only 3% of the activity observed for mercury-induced cells (data not presented).

Discussion

Plasmid pGB130 has been shown to promote its own transfer among strains of *B. cereus* and *B. thuringiensis*. Transfer of pGB130 and mercury resistance from *B. cereus* 5 to *B. cereus* 569 UM20-1 and *B. thuringiensis* 404A UM8td2 recipients occurred during mixed incubation on agar. Plasmid pGB130 did not promote transfer of two cryptic plasmids from *B. cereus* 5 All Hg^r *B. cereus* 569 UM20-1 transconjugants examined inherited pGB130 but all Hg^r *B. thuringiensis* 4042A UM8td2 transconjugants examined lacked pGB130. In addition, only *B. cereus* 569 UM20-1 Hg^r transconjugants were effective donors of pGB130 and the mercury-resistance trait, while *B. thuringiensis* 4042A UM8td2 Hg^r transconjugants were nonfertile. Isolation of nonfertile Hg^r derivatives of *B. thuringiensis* 4042A UM8td2 suggested pGB130, or the region coding for mercury resistance, may have inserted into the chromosome, and its presence may have inhibited or reduced the transfer ability of the self-transmissible plasmids pX011 and pX012 present in this strain.

Since the mechanism of plasmid transfer between *Bacillus* spp. is still unknown, there are several observations that support a conjugation-like system: (a) presence of DNase in mating mixtures did not reduce frequencies of transfer; (b) donor filtrates were inactive and cell-to-cell contact was required; and (c) the high frequencies of transfer (10^{-5} to 10^{-4}) are typical of conjugal transfer processes. Cell-to-cell contact and ineffectiveness of DNase in preventing transfer of pGB130 from *B. cereus* donors are findings similar to those of van Elsas and Pereira (1986), Battisti et al. (1985) and Gonzalez and Carlton (1982).

B. cereus 569 UM20-1 was successfully transformed by high-voltage electroporation with pGB130 isolated from both *B. cereus* 5 and a *B. cereus* 569 UM20-1 Hg^r transconjugant. Transformation frequencies ranged over 10^{-6}–10^{-5} with efficiencies between 10^3–10^4 Hg^r transformants/μg pGB130 DNA. All *B. cereus* 569 UM20-1 transconjugants harboring pGB130 were Hg^r and PMA^r, although *B. thuringiensis* 4042A UM8td2 Hg^r PMA^r transconjugants did not appear to inherit pGB130. All transformants obtained by electroporation of *B. cereus* 569 UM20-1 with pGB130 were Hg^r and PMA^r. Transconjugants and transformants expressed inducible levels of mercuric reductase activity when grown in the presence of either $HgCl_2$ or PMA. Induction of mercuric reductase by PMA indiated an additional organmercurial detoxifying enzyme, organomercurial lyase, was probably encoded by pGB130, and expressed in Hg^r PMA^r recipients. Only bacteria possessing broad-spectrum mercury determinants volatilize Hg^{2+} to Hg^0 upon induction by PMA (Jobling et al. 1988).

Plasmid pGB130 carries a selectable phenotype (Hg^r PMA^r) unique among the *B. cereus–B. thuringiensis* species group; it has the advantage over other phenotypic markers in naturally occurring bacilli (including protoxin-encoded plasmids of *B. thuringiensis* strains) of containing mercuric reductase activity that is simple to detect in presumptive recipients. Although pGB130 may be too large as a cloning vector in *Bacillus* research, it is one of a small number of naturally occurring plasmids in the genus *Bacillus* that possesses an

easily selectable phenotype. Most naturally occurring *Bacillus* plasmids used as cloning vectors are small, non-conjugative and carry either a bacteriocin or an antibiotic resistance marker, i.e. to erythromycin, tetracycline or chloramphenicol (Workman et al. 1986). Resistance to mercurials is a newly reported phenotype among native *Bacillus* plasmids.

The electroporation-induced transformation system employed in the present study provides an efficient method for interspecies transfer of *Bacillus* plasmids and has important applications in research with this genus. Transformation by electroporation is a useful artificial genetic-exchange technique for introducing cloning or expression vectors in *Bacillus,* particularly in industrially important species, since few have known conjugation, transformation or transduction systems (Workman et al. 1986). In addition, reproducible procedures for plasmid transformation must be developed, especially for large plasmids, since many procedure (i.e. protoplasting and competent cells, or transduction) are not suitable for such DNA molecules (Workman et al. 1986). Further characterzation of the genetic exchange mechanisms among *Bacillus* spp., particularly conjugation, will be useful in understanding plasmid biology in this genus.

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