Occurrence, Transfer and Mobilization in Epilithic Strains of Acinetobacter of Mercury-resistance Plasmids Capable of Transformation

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A 7.8 kb plasmid (pQM17) encoding mercury resistance was isolated from two epilithic strains of Acinetobacter calcoaceticus. The plasmid had a broad host range when mobilized by RP1, transferring into Pseudomonas aeruginosa, P. putida, P. fluorescens, Escherichia coli, Proteus vulgaris and Chromobacterium sp. with frequencies ranging from 5.3 × 10⁻⁹ to 4.6 × 10⁻⁴ per recipient. The plasmid could be transferred into A. calcoaceticus BD413 using intact cells of donor and recipient bacteria (i.e. natural transformation) and there was a broad temperature optimum (14-37 °C) for transformation. Transformation was as efficient in liquid matings as on plates but there was no effect of pH in the range 5.6-7.9. Maximum transformation frequencies were obtained after 24 h on agar plates containing 3.5-10 g C l⁻¹ with donor to recipient ratios ranging from 6 to 415.

INTRODUCTION

Most research on transferable resistances in bacteria isolated from natural environments has concentrated on conjugative plasmids (Kelly & Reaney, 1984; Khesin & Karasyova, 1984; Toranzo et al., 1984; Gauthier et al., 1985), but it has been suggested that the most common types of plasmid in bacteria are small (<20 kb) and therefore non-conjugative (Kelly & Reaney, 1984).

The epilithon is a heterogeneous slime community coating submerged surfaces in aquatic environments (Lock, 1981). Recent work in this laboratory has demonstrated the presence of large conjugative mercury-resistance plasmids in epilithic bacteria in rivers (Bale et al., 1987, 1988; Rochelle et al., 1989) but most plasmids in bacteria isolated from the epilithon were ≤20 kb (unpublished results). If these small plasmids are transferred it is likely to be by mobilization by a conjugative plasmid, by transduction, or by transformation.

Transformation of bacteria, artificially induced for competence, by chromosomal and plasmid DNA has been extensively studied, particularly in Pseudomonas spp. and Escherichia coli, and optimum conditions for transformation of these bacteria have been defined (Gross & Vidaver, 1981; Hanahan, 1985; Franklin, 1985; Berry & Kropinski, 1986). However, the artificial conditions necessary for the transformation of these bacteria are unlikely to occur in natural microbial habitats. Thus transformation will only be a likely mechanism for the transfer of genetic material in natural environments if bacteria which are naturally competent for transformation are present. Natural competence has been demonstrated in both Gram-positive (Graham & Istock, 1978; Morrison et al., 1982) and Gram-negative bacteria (Juni, 1972; Ahlquist et al., 1980; Albritton et al., 1982; Carlson et al., 1983). Natural transfer of DNA between intact donor and recipient cells by transformation has been reported in some bacteria (Stewart et al., 1983; Vakeria et al., 1985).

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Some strains of *Acinetobacter calcoaceticus* are naturally competent for transformation (Juni, 1972; Ahlquist et al., 1980) and *Acinetobacter* spp. are common in soil and water (Bauman, 1968; Juni, 1972; Kherson & Karasyova, 1984). It is therefore possible that *Acinetobacter* spp. could be active in promoting gene transfer in natural environments through transformation. The aim of this study was to investigate the occurrence of plasmids capable of transforming epilithic bacteria to mercury resistance, and to determine the influence of a variety of environmental variables on transformation.

**METHODS**

**Bacterial strains and growth conditions.** Two naturally competent strains of *A. calcoaceticus* were used, BD413 (NCIB 11826) (a prototroph) and NCIB 11827 (a histidine auxotrophic mutant of BD413). Epilithic bacteria were isolated from the River Taff, South Wales, and the four chosen *A. calcoaceticus* strains were designated PAR102 (Hg'), PAR108 (Hg'), PAR201 (Hg') and PAR206 Hg'). Each epilithic isolate contained at least one plasmid and was prototrophic. An epilithic isolate of *Chromobacterium* sp., AR34, was also used. Details of the other strains used are given in Table 1. Strains were routinely maintained on standard plate count agar (PCA; Oxoid CM643). Liquid cultures were grown in 10 ml nutrient broth (NB). Rifampicin-resistant mutants were used as recipients in natural transformation and conjugation experiments. The rifampicin-resistant mutant of BD413 is designated BD413R.

**Isolation of epilithic strains.** Stones were collected from a shallow area in the lower reaches of the organically polluted River Taff, South Wales (Mawle et al., 1985). Epilithic suspensions were prepared by scrubbing the stones followed by stomaching (Burton et al., 1982). Viable counts were performed on PCA at 20 °C for 4 d. For isolation of epilithic *Acinetobacter* spp., 1 ml volumes of suspensions of epilithic bacteria were inoculated into 5 ml of a mineral acetate enrichment medium (Bauman, 1968) and incubated at 20 °C for 4 d. Following incubation, samples were inoculated on PCA and PCA + HgCl₂ and incubated at 20 °C for 48 h. Colonies from these plates were replica-plated to a differential carbohydrate medium (LaCroix & Cabelli, 1982) and mineral acetate agar. The oxidase and Gram reactions of each of the isolates were also determined. Non-fermentative, oxidase-negative and Gram-negative isolates were presumed to be *Acinetobacter* spp.

**Identification of plasmids.** Crude cell lysates of epilithic *Acinetobacter* spp. were used to transform *A. calcoaceticus* BD413 and NCIB 11827 to mercury resistance and prototrophy using the method described by Juni (1972). Mercury-resistant transconjugants were examined for the presence of plasmids.

**Transformation.** Transformation from cell lysates was done as described above (Juni, 1972). Natural transformation between whole cells was done by the following procedures. Broth matings were performed by mixing 1 ml volumes of donor (plus pQM17) and recipient in 2 ml fresh NB and incubating for 24 h at 20 °C. Plate matings differed in that donor and recipient bacteria were deposited on 0.2 μm membrane filters and were incubated on PCA plates. Following incubation, cells were made up to or resuspended in 10 ml of 1/10-strength NB and enumerated by plate counts. Recipients and transformants were selected on PCA + rifampicin (100 μg ml⁻¹) + HgCl₂ (27 μg ml⁻¹), unless otherwise stated. All selective plates were incubated at 20 °C for 48 h. The transformation frequency was calculated as the number of transfectants per recipient. Throughout this work plasmid and chromosomal transfer were detected by selecting for mercury resistance and prototrophy, respectively. DNAase sensitivity was tested by adding DNAase I (0.4 pg ml⁻¹; Sigma) to the recipient culture prior to deposition on the membrane filter.

**Conjugation.** The broad-host-range plasmid RP1 was transferred from *E. coli* LE392 to BD413 containing pQM17 by membrane filter mating. Donor and recipient NB cultures (1 ml each) were mixed and filtered through 0.22 μm cellulose acetate membrane filters (25 mm diam.; Oxoid N25/22UP). The filters were incubated face up on the surface of PCA plates for 24 h at 30 °C. Following incubation, the cells on the filter were resuspended by vortex mixing in 10 ml 1/10-strength NB and serial dilutions were plated onto selective media. Transconjugants were selected on minimal salts agar (Bale et al., 1987) + sodium succinate (1 mg ml⁻¹) + carbenicillin (1 mg ml⁻¹) + HgCl₂ (13.5 μg ml⁻¹) incubated at 20 °C for 48 h. RP1 was then used to mobilize pQM17 into a range of recipient bacteria in membrane filter matings at 20 °C on PCA. Recipients and transconjugants were selected on PCA + rifampicin and PCA + rifampicin + HgCl₂. Selective plates were incubated at 20 °C for 48 h.

**DNA techniques.** Plasmids were detected using the method of Kado & Liu (1981) and their sizes estimated as described by Rochelle et al. (1986). Plasmid DNA for restriction digests was prepared by the method of Birnboim & Doly (1979). Restriction enzymes were obtained from Northumbria Biologicals and were used as recommended by the supplier.

**Statistical analysis.** One-way analysis of variance using SPSS-X (Hull & Nie, 1981) was used to compare differences between means. Minimum significant ranges (MSR) were calculated by the Tukey-HSD method (Sokal & Rohlf, 1981). Plasmid transformation frequencies were transformed by log₁₀ x to ensure homogeneous variances and normality of distribution.
RESULTS

Isolation and characterization of pQM17

Out of 100 random epilithic isolates, four were identified as *A. calcoaceticus*. Two of these isolates (PAR201 and PAR206) were mercury-resistant, and all four contained plasmids ranging in size from 2.5 kb to 175 kb (Fig. 1a). Crude cell lysates (Juni, 1972) of all four isolates transformed the auxotrophic strain NCIB 11827 to prototrophy, and lysates of PAR201 and PAR206 transformed NCIB 11827 and BD413 to mercury resistance. Mercury-resistant NCIB 11827 and BD413 transformants contained a single 7.8 kb plasmid (Fig. 1b) which was also seen in PAR201 and PAR206. Restriction digests, with EcoRI and HindIII, of the two plasmids (pQM17 and pQM18) transferred from PAR201 and PAR206 were indistinguishable (results not shown). The plasmids had one EcoRI and two HindIII sites but they were not digested by BamHI. Attempts to transform the two mercury-sensitive isolates, PAR102 and PAR108, to mercury resistance using crude lysates of PAR201 and PAR206 were unsuccessful.

The host range of pQM17 was determined using RP1 as the mobilizing plasmid (Table 1). pQM17 had a broad host range; it transferred into three *Pseudomonas* spp., *E. coli*, *Proteus vulgaris*, and an epilithic isolate of *Chromobacterium* sp. with frequencies ranging from

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**Fig. 1.** Gel electrophoresis of plasmid DNA in epilithic isolates of *A. calcoaceticus*. Plasmids were detected using the method of Kado & Liu (1981). Electrophoresis was carried out at 90 V for 1.5 h in 0.7% agarose. (a) Lanes: (1) *E. coli* V517(pVA517A-H; 55.1, 7.4, 5.6, 5.2, 4.1, 3.1, 2.7, 2.1 kb); (2) PAR102; (3) PAR108; (4) PAR201; (5) PAR206. (b) Lanes: *A. calcoaceticus* BD413(pQM17), transformed from (1) a crude cell lysate of PAR201 and (2) from intact donor cells of PAR201; (3) BD413R(pQM17) transformed from intact donor cells of BD413(pQM17); (4) BD413(pQM18), transformed from crude cell lysates of PAR206; (5) NCIB 11827(pQM17), transformed from crude cell lysates of PAR201; (6) NCIB 11827(pQM18), transformed from crude cell lysates of PAR206; (7) PAR201.
Table 1. Host range of pQM17

RP1 was used to mobilize pQM17 from BD413 to rifampicin-resistant mutants of each of the recipients. The transfer frequency was expressed as the number of mercury-resistant transconjugants per recipient and the presence of pQM17 in the transconjugants was confirmed by gel electrophoresis.

<table>
<thead>
<tr>
<th>Recipient bacterium</th>
<th>Genotype/phenotype*</th>
<th>Reference</th>
<th>Transfer frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acinetobacter calcoaceticus</td>
<td>BD413R Rif⁺ mutant of BD413</td>
<td>This paper</td>
<td>3.4 x 10⁻¹</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>PAO348 met-28 trp-6 lys-12 pro-82</td>
<td>V. Krishnapillai (pers. comm.)</td>
<td>1.2 x 10⁻⁶</td>
</tr>
<tr>
<td></td>
<td>PU21 ielB12 leu-1 str-1 Rif⁺</td>
<td>Jacoby (1974)</td>
<td>1.0 x 10⁻⁷</td>
</tr>
<tr>
<td>P. putida</td>
<td>KT2440 hsdR1 hsdM⁺; prototrophic</td>
<td>Bagdasarian et al. (1981)</td>
<td>1.1 x 10⁻⁶</td>
</tr>
<tr>
<td>P. fluorescens</td>
<td>AR41 Prototrophic; P⁻</td>
<td>Epilithic isolate</td>
<td>4.6 x 10⁻⁴</td>
</tr>
<tr>
<td></td>
<td>10038 Prototrophic</td>
<td>NTCT 10038</td>
<td>&lt;3.7 x 10⁻⁸†</td>
</tr>
<tr>
<td>P. maltophilia</td>
<td>AR27 Met⁺ P⁻</td>
<td>Epilithic isolate</td>
<td>&lt;5.0 x 10⁻⁸†</td>
</tr>
<tr>
<td></td>
<td>10499 Met⁻</td>
<td>NTCT 10499</td>
<td>&lt;3.4 x 10⁻⁸†</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>LE392 supE44 supF58 lacY1 galK2 metB1 trpR35 hsdR514 Δ⁻ F⁻</td>
<td>Maniatis et al. (1982)</td>
<td>4.5 x 10⁻⁴</td>
</tr>
<tr>
<td></td>
<td>J53 proB22 metF63</td>
<td>Bachmann (1987)</td>
<td>4.6 x 10⁻⁵</td>
</tr>
<tr>
<td>Proteus vulgaris</td>
<td>6213 Prototrophic</td>
<td>NCIB 6213</td>
<td>1.9 x 10⁻⁷</td>
</tr>
<tr>
<td>Chromobacterium sp.</td>
<td>AR34 Prototrophic</td>
<td>Epilithic isolate</td>
<td>5.3 x 10⁻⁹</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>8172 Prototrophic</td>
<td>NCIB 8172</td>
<td>&lt;8.9 x 10⁻⁸†</td>
</tr>
</tbody>
</table>

* P⁻, plasmid-free. † Lowest detectable frequency.

5.3 x 10⁻⁹ to 4.6 x 10⁻⁴. The transfer frequency of pQM17 in intra-strain matings using BD413 as donor and BD413R as recipient was 3.4 x 10⁻¹.

In an attempt to isolate more transforming plasmids from the epilithon, presumptive Acinetobacter spp. (Bauman, 1968; LaCroix & Cabelli, 1982) were used as sources of transforming DNA. Out of 14 mercury-resistant isolates, crude cell lysates of 10 transformed NCIB 11827 to prototrophy but none of them transformed either NCIB 11827 or BD413 to mercury resistance. These mercury-resistant Acinetobacter isolates contained plasmids ranging in size from 2.1 kb to 220 kb. Attempts to isolate Acinetobacter spp. from the epilithon that were naturally competent for transformation by pQM17 DNA were unsuccessful. None of the 75 mercury-sensitive isolates tested were transformed to mercury resistance.

Natural transformation by pQM17

Plasmid pQM17 transferred from whole cells of PAR201 to a rifampicin-resistant mutant of BD413 (BD413R) in membrane filter matings at a frequency of 2.5 x 10⁻⁷ per recipient. Transformants always contained pQM17 when examined by electrophoresis. Transfer did not occur in the presence of DNase (0.4 μg ml⁻¹) (frequency <1.0 x 10⁻⁸). This clearly shows that transfer was by transformation and not mobilization by the large plasmid indigenous to PAR201 (Fig. 1a; lane 4). The filtrate of an 18 h NB culture of PAR201 did not transform BD413 to mercury resistance or NCIB 11827 to prototrophy. Transfer of pQM17 occurred at a frequency of 6.0 x 10⁻⁴ when using BD413 as the donor and BD413R as the recipient. This transfer was also DNase I sensitive (transfer frequency in the presence of DNase I <1.0 x 10⁻⁸). Therefore, PAR201 and BD413 both behaved as natural donors for transformation by pQM17 without the need for cell lysis. Mutants of PAR201 and BD413 to rifampicin resistance occurred at <6 x 10⁻¹⁰ and <1 x 10⁻⁹ respectively. The mutation rate of BD413 to mercury resistance was <1.6 x 10⁻⁹ and of NCIB 11827 to prototrophy was <1.4 x 10⁻⁹.
Mercury-resistance plasmids in Acinetobacter

Fig. 2. Effect of temperature on transformation. Crude cell lysates were used to transfer pQM17 (○) and chromosomal DNA (▲) from PAR201 to NCIB 11827. Transformation was also carried out using intact donor and recipient cells to transfer pQM17 from BD413 to BD413R (●). Transformations (n = 2) were performed on PCA plates incubated for 24 h. MSR, minimum significant range. ▼, lowest detectable frequency, <8 x 10⁻⁹.

Factors affecting transformation

Crude lysates of PAR201 were used to transfer pQM17 into BD413; the temperature optimum for this process was 20-37 °C (Fig. 2; highest frequency = 2.3 x 10⁻⁷). The temperature optimum for the transformation of NCIB 11827 to prototrophy was 15-30 °C and the maximum frequency was 1.2 x 10⁻⁵. Fig. 2 also shows a broad temperature optimum (14-37 °C) for natural transfer of pQM17 in intra-strain transformations using intact donor and recipient cells. Using crude cell lysates transformation to prototrophy was detected at 8 °C (6.5 x 10⁻⁷) but not at 6 °C (<1.0 x 10⁻⁸) and transformation to mercury resistance was not detected below 10 °C. However, pQM17 could transfer by natural transformation at temperatures as low as 4.5 °C (frequency 1.2 x 10⁻⁷).

The effects of various other physico-chemical variables on natural transformation by pQM17 in intra-strain transfers using BD413 and BD413R were investigated. Transformation of pQM17 was not significantly affected by pH over the range pH 5.6-7.9 (Fig. 3a). Mercury-resistant transformants were detected after 0.5 h co-incubation of donor and recipient (frequency 2.5 x 10⁻⁸), but maximum frequencies were obtained after 24 h (Fig. 3b); increasing the incubation time to 48 h had no significant effect on the transformation frequency. Transfer was also influenced by the nutrient status of the medium (Fig. 3c). Transformation occurred on agar with no added carbon (frequency 4.0 x 10⁻⁶), but maximum frequencies were obtained on media containing 3.5-10 g C l⁻¹. Although transformation occurred at all initial donor to recipient ratios used it was maximal when an excess of donors over recipients was present (Fig. 3d). Natural transformation was as efficient in liquid matings (7.4 x 10⁻⁵ per recipient and 1.9 x 10⁻⁴ per donor) as on the surface of agar plates (1.0 x 10⁻⁴ per recipient and 7.8 x 10⁻⁵ per donor). One-way analysis of variance showed no significant difference (P < 0.05) between transfer frequencies.

DISCUSSION

The results showed that plasmids were common in natural Acinetobacter isolates (Fig. 1a) and that two independent isolates with different overall plasmid profiles contained similar 7.8 kb plasmids encoding mercury resistance and capable of transformation (pQM17 and pQM18).
Fig. 3. Factors affecting the natural transfer of pQM17 in intra-strain transformations using *A. calcoaceticus* BD413 as donor and BD413R as recipient. The variables examined were (a) pH of the transformation medium, (b) length of incubation time before plating on selective media, (c) nutrient status of the transformation medium, and (d) the initial donor to recipient ratio in the transformation mixture. MSR, minimum significant range.

Other *Acinetobacter* spp. from water (Olson et al., 1979) and soil (Lomovskaya et al., 1986) also contain small plasmids (about 7.5 kb) encoding mercury resistance and one of these, pKL1, was detected in isolates from three different soil samples (Lomovskaya et al., 1986). The plasmid pQM17 had a broad host range when mobilized by RP1 (Table 1) as did pKL1 (Lomovskaya et al., 1986). This suggests that small non-conjugative plasmids as well as conjugative plasmids (Kelly & Reanney, 1984; Khesin & Karasyova, 1984; Rochelle et al., 1989) might be important in the maintenance and spread of mercury resistance genes in natural bacterial populations. However, pQM17 was probably not abundant in epilithic bacteria because further attempts to isolate plasmids that were able to transform BD413 to mercury resistance were unsuccessful.

For natural transformation to be biologically significant, both donation and uptake of DNA should occur without being induced. The recipient used in this study was *A. calcoaceticus* BD413, a microcapsular mutant derived from a soil isolate, which is naturally competent for transformation (Juni, 1972). Although natural competence has also been demonstrated in another *A. calcoaceticus* strain (NCIB 8250; Ahlquist et al., 1980), it is not a common phenomenon. Juni (1978) reported that only two weakly competent strains were detected out of several hundred tested and we found no competent strains of *Acinetobacter* in the epilithon. Carlson et al. (1983) showed that natural competence in *Pseudomonas* spp. was restricted to *P. stutzeri* and closely related species; in these studies only double-stranded chromosomal DNA was active in transformation; plasmid DNA was not taken up.
We demonstrated the DNAase I-sensitive transfer of pQM17 from intact cells of both PAR201 and BD413 using BD413R as the recipient. Transformation-like processes involving whole cells of donors and recipients have also been reported in P. stutzeri (Stewart et al., 1983), Haemophilus influenzae (Albritton et al., 1982) and Bacillus subtilis (Graham & Istock, 1978). All this evidence suggests that transformation between intact cells may be a common phenomenon amongst bacteria.

The DNAase sensitivity of transformation by pQM17 from intact donors indicates that naked DNA must be present during transfer. It has been suggested that DNA may be released from bacteria into the environment (Reanney et al., 1983); and extracellular DNA is found in soil and water (Torsvik & Groksoyr, 1978; Paul & Myers, 1982). Therefore DNA is available in the environment, but it is susceptible to enzymic degradation. Forty percent (n = 50) of epilithic isolates we examined produced extracellular DNAase activity (unpublished results), which indicates that naked DNA may not survive for long in the epilithon. Aardema et al. (1983), however, reported that transforming DNA resisted enzymic degradation for longer when bound to sediment than in free solution. Transformation of B. subtilis also occurred in sand (Lorenz et al., 1988) and between intact donor and recipient cells in soil (Graham & Istock, 1978) in the presence of DNAase. Transformation may therefore occur in the environment despite the presence of nucleases.

Transformation by pQM17 and chromosomal DNA had a broad temperature optimum (Fig. 2), with transfer from intact donor cells being about 10³-fold more efficient than when using crude cell lysates. Relatively high transformation frequencies were obtained at 14 °C (6.3 x 10⁻⁶). As the average temperature of the River Taff over a 12 month period was 11 °C, this suggests that temperature would not be a limiting factor for plasmid transformation within the epilithon. The observation of highest transformation frequencies on media containing higher concentrations of organic nutrients (Fig. 3c) was consistent with reports that rapid growth of recipient cells promotes transformation (Cruze et al., 1979; Singer et al., 1986). Transformation of A. calcoaceticus with cell-free DNA preparations is dependent on the physiological status of the recipient and a peak of competence is reached during the early stages of exponential growth (Cruze et al., 1979; Ahlquist et al., 1980). Transformation frequencies with pQM17 were highest when there were more donors than recipients during the mating (Fig. 3d). This may have been because greater amounts of pQM17 DNA were available with excess donors, since it has been shown that transformation frequencies using cell-free DNA increase with rising DNA concentration, up to a point where the DNA becomes saturating (Cruze et al., 1979; Gross & Vidaver, 1981).

Our investigation demonstrated the presence of a plasmid encoding mercury resistance, which could be transferred by transformation and mobilization. Natural transformation occurred over a wide range of environmentally relevant conditions. However, only a few naturally competent bacteria have been isolated from microbial habitats, so transformation may only have a limited role in gene transfer in the natural environment.

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REFERENCES


