Directed introduction of DNA cleavage sites to produce a high-resolution genetic and physical map of the *Acinetobacter* sp. strain ADP1 (BD413UE) chromosome

Elizabeth M. Gralton,† Alan L. Campbell and Ellen L. Neidle

The natural transformability of the soil bacterium *Acinetobacter* sp. ADP1 (BD413UE), formerly classified as *A. calcoaceticus*, has facilitated previous physiological and biochemical investigations. In the present studies, the natural transformation system was exploited to generate a physical and genetic map of this strain’s 3780±191 kbp circular chromosome. Previously isolated *Acinetobacter* genes were modified *in vitro* to incorporate a recognition sequence for the restriction endonuclease NotI. Following transformation of the wild-type strain by the modified DNA, homologous recombination placed each engineered NotI cleavage site at the chromosomal location of the corresponding gene. This allowed precise gene localization and orientation of more than 40 genes relative to a physical map which was constructed with transverse alternating field electrophoresis (TAFE) and Southern hybridization methods. The positions of NotI, Ascl and I-CeuI recognition sites were determined, and the latter enzyme identified the presence of seven ribosomal RNA operons. Multiple chromosomal copies of insertion sequence IS1236 were indicated by hybridization. Several of these copies were concentrated in one region of the chromosome in which a spontaneous deletion of approximately 100 kbp occurred. Moreover, contrary to previous reports, ColEl-based plasmids appeared to replicate autonomously in *Acinetobacter* sp. ADP1.

Keywords: *Acinetobacter*, genome, map, PFGE (pulsed-field gel electrophoresis), TAFE (transverse alternating-field electrophoresis)

INTRODUCTION

Eubacteria of the genus *Acinetobacter*, classified in the γ-subdivision of the *Proteobacteria* (Rainey et al., 1994), are Gram-negative, strictly aerobic and ubiquitous in the environment (Baumann et al., 1968; Juni, 1978). *Acinetobacter* strains can degrade a wide range of organic compounds including aliphatic alcohols, amino acids, dicarboxylic and fatty acids, alkanes and many aromatic compounds (Juni, 1978). The potential use of bacteria for bioremediation has led to renewed interest in the catabolic abilities of *Acinetobacter* strains. Renewed interest in *Acinetobacter* physiology also stems from the ability of some isolates to cause nosocomial infections, especially those associated with immunocompromised patients (Bergogne-Bérézin, 1994).

Epidemiological investigations have spearheaded new taxonomic studies (Gerner-Smidt, 1994). *Acinetobacter* classification has historically been confusing due to the nondescript phenotype of this genus and the misidentification of individual strains. Strains now known to be acinetobacters were originally classified into more than 10 different genera (Towner et al., 1991). One method for identification of *Acinetobacter* strains involves the transformation of a particular *trpE* auxotroph. DNA from only *Acinetobacter* strains can transform the *trpE* auxotroph to prototrophy (Juni, 1972). This *trpE* auxotroph is an ideal recipient strain because it is naturally competent for transformation.

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Abbreviation: TAFE, transverse alternating-field electrophoresis.
Only strains which can trace their lineage to the encapsulated soil isolate BD4 (Juni & Janik, 1969) have been found to be naturally competent. ADP1, also designated BD413UE, is an unencapsulated (UE), naturally competent derivative of BD4 that was an intermediate in the isolation of the trpE auxotroph (Juni & Janik, 1969). ADP1 has been the subject of numerous investigations, including studies of aromatic compound degradation (Ornston & Neidle, 1991; Harwood & Parales, 1996). The natural competence of ADP1 has enabled a genetic approach to biochemical and physiological questions that could not have been readily addressed in other ways. This mapping project was undertaken to further the development of genetic techniques. In these studies, exploitation of the natural transformation system of ADP1 was central to the precision with which genes could be localized.

Modified plasmid DNA was used to transform Acinetobacter recipient strains and introduce novel restriction endonuclease recognition sites in the chromosome. The positions of these recognition sites were determined by a combination of Southern hybridization techniques and a type of pulsed-field gel electrophoresis, transverse alternating field electrophoresis (TAFE). Mapping studies will contribute to ongoing investigations of Acinetobacter genetic organization, function and evolution. In previous studies, RP4-mediated conjugation was used to determine the relative locations of 23 different markers on a linkage map of A. calcoaceticus strain EB65/65 (Towner, 1978). Although circulation of the chromosome was demonstrated, the gene transfer frequency of this method was low, and it was not possible to deduce the distance between the markers (Towner et al., 1991). The methods used here not only demonstrated the circularity and size of the ADP1 chromosome, but allowed gene orientation on a high-resolution correlated physical and genetic map.

**METHODS**

**Strains, plasmids and growth conditions.** The wild-type Acinetobacter sp. strain ADP1 is the unencapsulated BD4 (ATCC 33305) isolated by Juni & Janik (1969). The taxonomic classification of this strain, formerly A. calcoaceticus, has been called into question (Strat et al., 1996), prompting the use of Acinetobacter sp. until further characterization is undertaken. ADP1-derived mutants and plasmids are listed in Table 1. Escherichia coli plasmid-host strains DH5α (Bethesda Research Laboratories), JM109 (Yanisch-Perron et al., 1985) and Top10F* (Invitrogen) were used. The sources of the QKm and QSmSp cassettes were pU11637 and pU11638, respectively (Eraso & Kaplan, 1994). Bacteria were grown at 37 °C with aeration in Luria–Bertani broth (LB) or succinate minimal medium supplemented with antibiotics (Sambrook et al., 1989; Shanley et al., 1986).

**DNA manipulations and construction of mutant strains.** Standard methods were used for plasmid and chromosomal DNA purification, ligations, E. coli competent cell preparations and E. coli transformations (Sambrook et al., 1989). Purification kits (Qiagen or GeneClean from Bio101) were used to extract DNA fragments from agarose. Restriction enzymes were purchased from Promega, New England Biolabs or Boehringer Mannheim. NotI, I-CeuI and AecI were from New England Biolabs.

Plasmids carrying Δ-disrupted regions of Acinetobacter sp. DNA were linearized with restriction endonucleases and used to alter the ADP1 chromosome as previously described (Neidle et al., 1989). In some cases, a mutation in one Acinetobacter strain was incorporated into the chromosome of a second strain by making a crude DNA lysate of the first strain, by the method of Juni (1972), and using it to transform the second strain. This method was used to transfer the Tn5-disrupted epsX allele from strain BD4:171 (Stark, 1996) to the ADP1 chromosome, generating ACN16.1 (Table 1). Following the selection of mutants by antibiotic resistance, Southern hybridization methods confirmed chromosomal configurations.

**Southern hybridization analyses.** A rapid downward transfer system (TurboBlotter; Schleicher and Schuell) was used for Southern hybridizations (Sambrook et al., 1989) to transfer DNA to Nytran nylon membranes. To facilitate the transfer of large DNA fragments, acid depurination was used and followed by base cleavage according to the manufacturer’s instructions (Schleicher and Schuell). The only variation from suggested protocols was the use of a long transfer time, 18–24 h. Following DNA transfer, DNA was cross-linked to the membranes by exposure to a total dose of UV light (254 nm) of 120 mJ cm\(^{-2}\).

Nonradioactive probes were prepared by labelling DNA fragments with digoxigenin and a random-primed labelling system (Genius System, Boehringer Mannheim). Prehybridization, hybridization (at 42 °C in 50%, v/v, formamide) and high-stringency washes were done according to the manufacturer’s instructions (Boehringer Mannheim). These instructions were also followed for the detection of probes with anti-digoxigenin alkaline phosphatase conjugates and chemiluminescent substrates.

**Preparation of intact genomic DNA and restriction digestion for TAFE analysis.** Genomic DNA was prepared, from cells grown to stationary phase, in agarose plugs by the method of Smith & Cantor (1987). Restriction endonuclease digests were performed on the agarose-embedded DNA, using 8–15 units of enzyme with its corresponding commercial restriction buffer. Approximately one-eighth of the gel plug was sliced and equilibrated on ice with buffer and enzyme as described by Suwanto & Kaplan (1989). Digests were incubated for 6–8 h with gentle shaking (5–10 r.p.m.). Digests with two restriction endonucleases were done sequentially. After the initial digestion, the buffer/ enzyme mixture was removed and replaced with 300 μl 1× TE (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) and placed on ice for 15 min. This procedure was repeated four times before the second restriction endonuclease buffer was added and allowed to equilibrate on ice. After digestion, the buffer/ enzyme mixture was removed and 200 μl ESP solution (Smith & Cantor, 1987) was added, incubated at 55 °C for 10–15 min and then removed. The plug was placed in 300 μl 1× TE for 20–30 min at 4 °C prior to being placed in the well of the electrophoresis gel.

**TAFE conditions.** TAFE (Geneline II, Beckman) gels were formed from either 0.7%, low-melting-point agarose (SeaPlaque from FMC Bioproducts) or 1% agarose (Seakem GTG from FMC Bioproducts). For low-melting-point gel electrophoresis, the running buffer temperature was 7 ± 1 °C, while the 1% agarose gels were maintained at 12 ± 1 °C. DNA fragments of known sizes were used as standards. Yeast chromosomal standards (Promega), a low-range lambda concatemeric ladder, 1\(^{st}\) (New England BioLabs), and the
Table 1. Mapped genes, hybridization probes, relevant plasmids and mutant strains

<table>
<thead>
<tr>
<th>Locus</th>
<th>Gene product(s)</th>
<th>Relevant plasmid(s)</th>
<th>ADP1-derived mutant(s)</th>
<th>Reference(s) or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>alkr, alkm, orfy</td>
<td>Alkane degradation</td>
<td>pWH785, pBAC72, QKm in orfy near alkr</td>
<td>ACN67 (orfY1::QKm)</td>
<td>A. Ratajczak &amp; W. Hillen¹</td>
</tr>
<tr>
<td>benM</td>
<td>Regulator of benzoate degradation</td>
<td>pBAC11, pBAC58, QSpSm in benM SalI site</td>
<td>ACN53 (benM2::QSpSm)</td>
<td>L. Collier &amp; E. Neidle</td>
</tr>
<tr>
<td>catA</td>
<td>Catechol oxygenase</td>
<td>pB1343</td>
<td>ACN55 (benM2::QSpSm, recA::Tn5)</td>
<td>Neidle et al. (1987)</td>
</tr>
<tr>
<td>catM</td>
<td>Regulator of catechol degradation</td>
<td>pB15, pBAC6A, QKm in catM HincII site</td>
<td>ACN6.1 (catM1::QKm)</td>
<td>Neidle et al. (1989)</td>
</tr>
<tr>
<td>epsX, epsM</td>
<td>Exopolysaccharide formation</td>
<td>pNP2345</td>
<td>ACN16.1 (epsX::Tn5)</td>
<td>Stark (1996)</td>
</tr>
<tr>
<td>estA, rotA</td>
<td>Esterase, isomerase</td>
<td>pAKA24-5, pBAC69, QKm in estA Clal site</td>
<td>ACN64 (estA1::QKm)</td>
<td>Kok (1995), Kok et al. (1993)</td>
</tr>
<tr>
<td>estB, estR</td>
<td>Lipase, regulator</td>
<td>pAKA22-20, pBAC59, QSpSm in estR PmlI site</td>
<td>ACN45 (estR1::QSpSm)</td>
<td>Kok (1995), Kok et al. (1993)</td>
</tr>
<tr>
<td>mucK</td>
<td>cis,cis-Muconate transporter</td>
<td>pADPW1, pBAC101, QSpSm in mucK ΔNsiI site</td>
<td>ACN79 (mucK1::QSpSm)</td>
<td>P. A. Williams²</td>
</tr>
<tr>
<td>pca genes</td>
<td>Protocatechuate degradation</td>
<td>pZR6</td>
<td>ADP212 (pcaD104::Tn5)</td>
<td>Doten et al. (1987), Gregg-Jolly &amp; Ornston (1990)</td>
</tr>
<tr>
<td>pobA</td>
<td>4-Hydroxybenzoate hydroxylase</td>
<td>pZR405</td>
<td></td>
<td>Averhoff et al. (1992)</td>
</tr>
<tr>
<td>quiABC</td>
<td>Quinate degradation</td>
<td>pZR304</td>
<td></td>
<td>Elsemore &amp; Ornston (1994)</td>
</tr>
<tr>
<td>recA</td>
<td>RecA protein</td>
<td>pZR106</td>
<td>ADP197 (recA::Tn5)</td>
<td>Gregg-Jolly &amp; Ornston (1994)</td>
</tr>
<tr>
<td>rpoN</td>
<td>RNA polymerase σ⁴ subunit</td>
<td>pWH837, pBAC71, QKm in rpoN SnaBI site</td>
<td>ACN66 (rpoN1::QKm)</td>
<td>F. Schirmer, B. Argauer &amp; W. Hillen¹</td>
</tr>
<tr>
<td>rubAB</td>
<td>Alkane degradation</td>
<td>pWH981</td>
<td></td>
<td>Geissdörfer et al. (1995)</td>
</tr>
<tr>
<td>Tn5</td>
<td>Transposon</td>
<td></td>
<td>ISA1000.3, ISA1000.3 (Tn5 in Norl fragment C, Tn5 in Norl fragment A)</td>
<td>G. L. Gaines³</td>
</tr>
<tr>
<td>trpFB</td>
<td>Tryptophan biosynthesis</td>
<td>pJK15, pBAC46, QSpSm in BspEI site upstream of trpF</td>
<td>ACN27 (trp-l::QSpSm)</td>
<td>Ross et al. (1990)</td>
</tr>
<tr>
<td>trpGDC</td>
<td>Tryptophan biosynthesis</td>
<td>pBN78, pBAC51, QSpSm in trpD PstI site</td>
<td>ACN30 (trpD1::QSpSm)</td>
<td>Kaplan et al. (1984)</td>
</tr>
<tr>
<td>trpE</td>
<td>Tryptophan biosynthesis</td>
<td>pWH1705, pBAC95, QKm in trpE ΔClaI site</td>
<td>ACN74 (trpE1::QKm)</td>
<td>Haspel et al. (1990)</td>
</tr>
</tbody>
</table>

¹ pBAC plasmids and ACN strains were constructed for these studies. Affiliations of sources: 1, Friedrich-Alexander-Universität, Erlangen-Nürnberg, Germany; 2, University of Wales, Bangor, UK; 3, Isogenetics, Inc., Chicago, IL, USA.
lambda 39 concatemeric ladder, $\lambda_{39}$ (Promega) provided a wide range of fragment sizes.

Three protocols were used with different pulse and run times to separate DNA fragments of various sizes. An 18 h protocol with two stages maximized resolution of 500–1500 kbp fragments. The first stage used 350 mA of constant current with a pulse time of 45 s for 12 h. The second used 370 mA of current with a pulse time of 90 s for 6 h. A 26 h protocol, maximizing resolution of 50–500 kbp fragments, used four stages: (1) 350 mA, 30 s pulses, 5 h; (2) 370 mA, 45 s pulses, 8 h; (3) 370 mA, 60 s pulses, 8 h; (4) 390 mA, 90 s pulses, 5 h. A 30 h protocol maximized separation of fragments in the 10–300 kbp range and used four stages: (1) 350 mA, 20 s pulses, 6 h; (2) 350 mA, 30 s pulses, 12 h; (3) 370 mA, 45 s pulses, 10 h; (4) 370 mA, 60 s pulses, 2 h.

RESULTS

Selection of restriction enzymes and genome size

The restriction endonucleases NotI and Ascl, with G+C-rich recognition sequences, cleaved the A+T-rich chromosome of ADP1 into six and sixteen DNA fragments, respectively. I-CeuI, with a 26-nucleotide recognition sequence (Liu et al., 1993), cleaved the genome into seven fragments. DNA fragments between 5 and 1500 kbp were separated using the GeneLine II TAFE system (Beckman) with three different protocols (see Methods). Analysis of undigested wild-type genomic DNA was consistent with a single chromosome with no endogenous plasmids (data not shown).

Genomic fragments generated by digestion with NotI and I-CeuI were designated A to F and I to VII, respectively (Fig. 1, Table 2). Although similar use of Ascl resulted in 11 distinct bands (Fig. 1c), the relative intensities indicated that some represented more than one DNA fragment. Bands 6 (192 kbp), 9 (77 kbp) and 11 (23 kbp) may each correspond to two distinct DNA fragments, and band 8 (113 kbp) may represent three fragments. The genome size was deduced from the sizes of individual fragments following restriction endonuclease cleavage (Table 2). Fragment sizes were based on the means of 102 digests of the wild-type strain with NotI, 32 with I-CeuI and 27 with Ascl. Analyses of mutant strains, described below, were also included in the estimation of a 3780±191 kbp chromosome.

Construction of the physical map; location of NotI-generated fragments

Incomplete cleavage of genomic DNA with NotI generated four DNA fragments in addition to fragments A to F. Three were intermediate in size between fragments C and D, indicating that fragments D, E and F were adjacent on the chromosome. The sizes were consistent with the chromosomal order F-D-E. The fourth fragment identified by partial digestion was larger than C and smaller than B. Its size was equal to the sum of E and C, suggesting the chromosomal fragment order F-D-E-C.

Fragments A and B were located relative to F-D-E-C by using small DNA regions at the ends of the six NotI fragments as hybridization probes. These small fragments were isolated by ligating chromosomal DNA and cloning vector pSL301 (Invitrogen) which had both been cleaved with NotI and the more frequently cutting HindIII. Individual recombinant plasmids were isolated, and the ADP1 DNA inserts, designated junction fragments (JF), were purified. Using Southern hybridization methods, two of these fragments, JF312 and JF306, hybridized to fragment A. JF312 hybridized to I-CeuI fragment II (Fig. 2b), as did probes made from the entire fragments C or A. Use of the JF312 probe with genomic DNA digested by NotI and I-CeuI established the relative positions of fragments C, A and II (Fig. 2b).

JF306 hybridized to I-CeuI fragment I (Fig. 2a) and to Ascl fragment 3 (summarized in Table 3 and Fig. 3). Since Ascl fragment 3 hybridized to both fragments A and B, and since JF306 hybridized to a 68 kbp chromosomal fragment generated by cleavage with NotI and I-CeuI, the relative fragment positions were established (Figs 2a and 3). The NotI fragment order was therefore F-D-E-C-A-B. Additional hybridization results established that B and F are adjacent on the chromosome. For example, DNA at the end of NotI fragment B, JF311, hybridized to Ascl fragment 2, and labelled Ascl fragment 2 hybridized to both fragments B and F (Table 3, Fig. 3). The order of fragments is depicted on the physical map (Fig. 4).

Location of I-CeuI and Ascl-generated fragments on the physical map

The assigned locations of I-CeuI I and II (Fig. 4) were consistent with the TAFE patterns after sequential NotI and I-CeuI cleavage (Fig. 2). The positions of I-CeuI fragments III, IV, V, VI and VII, all internal to NotI fragment A, were determined with gene probes and mutant strains (Table 1). A probe made to the 615 kbp DNA region between the epsX gene and the end of fragment A (map positions 827–1442) hybridized to I-CeuI fragments II, IV and V. Probes made from the trpF and trpE markers hybridized to IV, and an epsX probe hybridized to V. The JF312 probe hybridized to the 38 kbp DNA region between map positions 1404 (trpF) and 1442, thus establishing the positions of fragments IV and V (Fig. 4). A probe from a region of fragment A in mutant ISA1000.5, map positions 0–421, hybridized to both fragments VI and III. A lipBA gene probe hybridized to fragment III. A probe made from the largest Ascl fragment (1) hybridized strongly to I-CeuI fragments VI, III, VII and V, and weakly to IV. The positions of all the I-CeuI fragments and some of the Ascl fragments were thereby determined (Table 3, Fig. 4).

The genetic map: generating mutants with new chromosomal NotI sites

Interposon mutagenesis introduced NotI recognition sequences at known locations in *Acinetobacter* sp. DNA. An Ω cartridge conferring resistance to streptomycin and spectinomycin (SpSm) or kanamycin (Km) was
inserted into Acinetobacter DNA carried on a recombinant plasmid. Each cartridge, derived from plasmid pUI1637 or pUI1638 (Eraso & Kaplan, 1994), carried a NotI restriction site adjacent to the drug-resistance determinant. The ΩSpSm cartridge was inserted in lipA, generating plasmid pBAC56 (Fig. 5a). Plasmids pBAC46, pBAC59, pBAC58, pBAC101 and pBAC51 were constructed by insertion of the RSpSm cartridge upstream of trpF, within estR, within benM, within mucK and within trpD, respectively (Table 1). The ΩKm cartridge was inserted into estA, trpE, rpoN, catM and upstream of alkR, forming pBAC69, pBAC95, pBAC71, pBAC6A and pBAC72 (Table 1).

These pBAC plasmids were individually linearized with a restriction endonuclease and used to transform strain ADP1. Mutants with a chromosomal copy of an Ω cartridge were selected by drug resistance. Each drug-resistant mutant had more than six genomic NotI DNA fragments (Fig. 6). The modified DNA appeared to have integrated in the homologous wild-type chromosomal region. For example, a labelled lipA probe (Fig. 5) hybridized to the wild-type NotI fragment A. In the lipA mutant ACN44, fragment A was cleaved into two smaller fragments, A1 (1100 kbp) and A2 (320 kbp) (Figs 5 and 6). The lipA probe hybridized to A2, consistent with the engineered NotI site being in the 5’ region of lipA. The smallest fragment generated by digestion of ACN44 genomic DNA with NotI (Fig. 6) corresponded to plasmid DNA, as discussed later.

Probes from catM, benM or alkR hybridized to NotI fragment E. In the corresponding mutants ACN6.1, ACN53 or ACN67, fragment E (210 kbp) was cleaved by NotI into two smaller fragments, E1 and E2 (Fig. 6). E1 and E2 were 170 and 40 kbp in ACN6.1, 160 and 50 kbp

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**Table 2. Acinetobacter sp. ADP1 DNA fragment sizes**

<table>
<thead>
<tr>
<th>Digestion with NotI</th>
<th>Digestion with I-CeuI</th>
<th>Digestion with Ascl</th>
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</thead>
<tbody>
<tr>
<td><strong>Fragment designation</strong></td>
<td><strong>Size (kbp)</strong></td>
<td><strong>Fragment designation</strong></td>
</tr>
<tr>
<td>A</td>
<td>1442 ± 55</td>
<td>I</td>
</tr>
<tr>
<td>B</td>
<td>1090 ± 27</td>
<td>II</td>
</tr>
<tr>
<td>C</td>
<td>653 ± 22</td>
<td>III</td>
</tr>
<tr>
<td>D</td>
<td>280 ± 23</td>
<td>IV</td>
</tr>
<tr>
<td>E</td>
<td>210 ± 24</td>
<td>V</td>
</tr>
<tr>
<td>F</td>
<td>105 ± 11</td>
<td>VI</td>
</tr>
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<td></td>
<td></td>
<td>VII</td>
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<tr>
<td><strong>Total:</strong></td>
<td><strong>3780 ± 162</strong></td>
<td><strong>3782 ± 221</strong></td>
</tr>
</tbody>
</table>
Fig. 2. Southern hybridization analysis of the relative positions of Nod fragments B, A and C. (a). Wild-type DNA digested with the enzymes indicated (TAFE gel, left) was hybridized to a JF306 probe. Solid arrows show the sizes (in kbp) of DNA fragments corresponding to the resultant hybridization signals (right). (b). As in (a) except the JF312 probe was used, DNA from the opposite end of fragment A. Additional experiments distinguished l-CeuI fragments I and II (not shown).

Fig. 3. Representations of Nodl fragment junctions. Letters enclosed in boxes indicate the two Nodl fragments depicted in each diagram. The relative positions of restriction fragments were deduced from hybridization data (Table 3).
Table 3. Summary of hybridization studies of the *NotI* fragment junctions

<table>
<thead>
<tr>
<th><em>NotI</em> junction</th>
<th>Labelled probe</th>
<th>Target DNA* cleaved by</th>
<th>Hybridization detected to</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-C</td>
<td>JF312</td>
<td><em>NotI</em></td>
<td><em>NotI</em> fragment A</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>I-CeuI</em></td>
<td><em>I-CeuI</em> fragment II</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>NotI</em> and <em>I-CeuI</em></td>
<td>10 kbp fragment</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Ascl</em></td>
<td><em>Ascl</em> fragment 8 (113 kbp)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>NotI</em> and <em>Ascl</em></td>
<td>13 kbp fragment</td>
</tr>
<tr>
<td></td>
<td><em>NotI</em> fragment C</td>
<td><em>I-CeuI</em></td>
<td><em>I-CeuI</em> fragment II</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>NotI</em> and <em>I-CeuI</em></td>
<td><em>NotI</em> fragment C</td>
</tr>
<tr>
<td>C-E</td>
<td>JF321</td>
<td><em>NotI</em> (ACN16.1)</td>
<td><em>NotI</em> fragment E2</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Ascl</em></td>
<td><em>Ascl</em> fragment 2</td>
</tr>
<tr>
<td>Fragment C2 of</td>
<td></td>
<td><em>Ascl</em></td>
<td><em>Ascl</em> fragments 5, 8</td>
</tr>
<tr>
<td>ISA1000.3</td>
<td></td>
<td><em>NotI</em> and <em>Ascl</em></td>
<td><em>Ascl</em> fragment 5, 100 kbp fragment</td>
</tr>
<tr>
<td>E-D</td>
<td><em>recA</em></td>
<td><em>NotI</em></td>
<td><em>NotI</em> fragment E</td>
</tr>
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<td></td>
<td></td>
<td><em>I-CeuI</em></td>
<td><em>I-CeuI</em> fragment II</td>
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<tr>
<td></td>
<td></td>
<td><em>Ascl</em></td>
<td><em>Ascl</em> fragment 7</td>
</tr>
<tr>
<td></td>
<td>JF321</td>
<td><em>Ascl</em></td>
<td><em>Ascl</em> fragment 2</td>
</tr>
<tr>
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<td></td>
<td><em>NotI</em> and <em>Ascl</em></td>
<td>77 kbp fragment</td>
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<td></td>
<td><em>NotI</em> fragment D</td>
<td><em>I-CeuI</em></td>
<td><em>I-CeuI</em> fragments I and II</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>NotI</em> and <em>I-CeuI</em></td>
<td>210 and 70 kbp fragments</td>
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<td></td>
<td><em>NotI</em> fragment E</td>
<td><em>Ascl</em></td>
<td><em>Ascl</em> fragments 2 and 7</td>
</tr>
<tr>
<td>I-CeuI fragment II</td>
<td><em>NotI</em></td>
<td><em>NotI</em></td>
<td><em>NotI</em> fragments C, D and E</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>NotI</em> and <em>I-CeuI</em></td>
<td><em>NotI</em> fragments C, E and 210 kbp portion of D</td>
</tr>
<tr>
<td>D-F</td>
<td><em>psaCHG</em></td>
<td><em>NotI</em></td>
<td><em>NotI</em> fragment D</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>I-CeuI</em></td>
<td><em>I-CeuI</em> fragment 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>NotI</em> and <em>I-CeuI</em></td>
<td>70 kbp fragment</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Ascl</em></td>
<td><em>Ascl</em> fragment 2</td>
</tr>
<tr>
<td></td>
<td><em>NotI</em> fragment D</td>
<td><em>I-CeuI</em></td>
<td><em>I-CeuI</em> fragments I and II</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>NotI</em> and <em>I-CeuI</em></td>
<td>210 and 70 kbp fragments</td>
</tr>
<tr>
<td></td>
<td><em>Ascl</em> fragment 2</td>
<td><em>NotI</em></td>
<td><em>NotI</em> fragments B, D, E and F</td>
</tr>
<tr>
<td>I-CeuI fragment II</td>
<td><em>NotI</em></td>
<td><em>NotI</em></td>
<td><em>NotI</em> fragments C, D and E</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>NotI</em> and <em>I-CeuI</em></td>
<td>650 and (2) 210 kbp fragments</td>
</tr>
<tr>
<td>F-B</td>
<td><em>Ascl</em> fragment 2</td>
<td><em>NotI</em></td>
<td><em>NotI</em> fragments B, D, E and F</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>NotI</em> (ACN30)</td>
<td><em>NotI</em> fragments B1, D, E and F</td>
</tr>
<tr>
<td></td>
<td>JF311</td>
<td><em>NotI</em></td>
<td><em>NotI</em> fragment B</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Ascl</em></td>
<td><em>Ascl</em> fragment 2</td>
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<td></td>
<td></td>
<td><em>NotI</em> and <em>Ascl</em></td>
<td>240 kbp fragment</td>
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<tr>
<td>B-A</td>
<td>JF306</td>
<td><em>NotI</em> and <em>I-CeuI</em></td>
<td>68 kbp fragment</td>
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<td><em>I-CeuI</em></td>
<td><em>I-CeuI</em> fragment 1</td>
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<td></td>
<td></td>
<td><em>Ascl</em></td>
<td><em>Ascl</em> fragment 3</td>
</tr>
<tr>
<td></td>
<td><em>NotI</em> fragment A</td>
<td><em>I-CeuI</em></td>
<td>All seven <em>I-Ceu</em> fragments</td>
</tr>
<tr>
<td></td>
<td><em>Ascl</em> fragment 3</td>
<td><em>NotI</em></td>
<td><em>NotI</em> fragments A and B</td>
</tr>
<tr>
<td>Fragment B2 of</td>
<td></td>
<td><em>I-CeuI</em></td>
<td><em>I-CeuI</em> fragment I</td>
</tr>
<tr>
<td>ACN30</td>
<td>trpGDC</td>
<td><em>Ascl digest</em></td>
<td><em>Ascl</em> fragments 3 and 8</td>
</tr>
</tbody>
</table>

* Wild-type target DNA except where parentheses indicate mutant strains.
Fig. 4. Physical and genetic map of the chromosome of Acinetobacter sp. strain ADP1 (BD413UE). The origin of the map (0/3780) was arbitrarily located at the NotI fragments B and A junction; position numbers indicate the distance (in kbp) from this origin. Arrows indicate the 5' to 3' orientations of genes.

Fig. 5. Representations of lipA on recombinant plasmids (a) or chromosomal fragments (b). Insertion of the $\Omega$SpSm cartridge (triangle) into the lipA EcoRI site yielded plasmid pBAC56 (a) and mutant ACN44 (b), each with a NotI site in the 5' end of lipA. A probe to the 3' end of lipA (a) indicated the chromosomal orientation of the gene (open arrow in b). Not drawn to scale.

in ACN53, and 140 and 70 kbp in ACN67. The smallest NotI DNA fragments of ACN53 and ACN67 (Fig. 6) corresponded to plasmid DNA, as discussed in subsequent sections. Moreover, in ACN67, the observed size of fragment C (C") was anomalously low. This size variation appeared to result from a spontaneous deletion in the wild-type strain rather than from insertion of a novel NotI recognition site. Although the wild-type NotI fragment C was determined to be 653 kbp, a change was noted in its size during these studies, resulting in the observed size of 550 kbp in some strains (C", Fig. 6). A spontaneous deletion in a laboratory isolate of ADP1 (lane 1, Fig. 6) could have been maintained in mutants which were constructed using the deleted wild-type strain as the recipient for transformation by modified DNA.

Labelled DNA from trpF, estA, rpoN or trpE hybridized to the wild-type fragment A. DNA from the corresponding mutants ACN27, ACN64, ACN66 and ACN74 yielded fragments A1 and A2 after NotI cleavage (Fig. 6). In the rpoN mutant ACN66 and in ACN27 (O near trpF) the A1 fragments were close in size to A and the A2 fragments were 5 and 40 kbp, respectively. In the estA mutant ACN64, A1 and A2 were 740 and 700 kbp, and in the trpE mutant ACN74 they were 1060 and 380 kbp. Two of these strains, ACN66 and ACN74, were found to have the smaller fragment C" rather than the expected 653 kbp fragment C.
The trpD, estR or mucK probes hybridized to wild-type NotI fragments B, C or D respectively. The sizes of B1 and B2 in ACN30 were 600 and 490 kbp, those of C1 and C2 in ACN45 were 430 and 220 kbp, while those of D1 and D2 in ACN79 were 240 and 40 kbp (Fig. 6). ACN45 appeared to carry a plasmid, whereas ACN79 had the 550 kbp fragment C*.

In strains ADP197, ADP212 and ACN16.1 (Table 1), the recA, pcaD or epsX genes are disrupted by Tn5, which contains NotI recognition sites. DNA probes made from the recA, pcaD or epsX genes hybridized to wild-type NotI fragments E, D or A respectively. In the recA mutant, ADP197, NotI cleavage generated two approximately 100 kbp fragments, E1 and E2, that were indistinguishable in size from fragment F in Fig. 6. D1 and D2 (250 and 30 kbp) were generated in the pcaD mutant, ADP212. A1 and A2 (830 and 615 kbp) were generated in the epsX mutant, ACN16.1 (Fig. 6).

**Relative orientations of genes**

The location and transcriptional direction of lipA were determined by hybridization of a lipA probe to wild-type NotI fragment A, I-CeuI fragment III and to NotI fragment A2 of ACN44 (Fig. 5). Probes from the 5' end of epsX or a region upstream of trpE hybridized to fragment A1 of ACN16.1 or ACN74, respectively, indicating gene orientations. Additional orientations (Fig. 4) were deduced from the following information. Probes from the 3' region of trpF or the 5' end of rpoN hybridized to the A2 fragments of ACN27 or ACN66. Probes downstream of alkR or catM hybridized to E1 of ACN67 or ACN6.1, respectively. Probes to the 5' region of mucK, or to a region downstream of pcaD, hybridized to D2 of ACN79 or ADP212, respectively.

A new strain was constructed to orient estA since this gene was close to the centre of fragment A. In ACN65, both the estA ΩKm cartridge and the lipA ΩSpSm cartridge were chromosomally inserted. NotI cleavage of this strain's DNA generated three smaller fragment A pieces. A probe from the 3' region of estA hybridized to the DNA between lipA and estA, establishing the estA orientation. Similarly, NotI digestion of ADP197 indicated that recA was in the centre of fragment E. A new strain, ACN55, was constructed with benM disrupted by the ΩSpSm cartridge and recA disrupted by Tn5. A probe from the 5' region of recA hybridized to the DNA fragment between benM and recA in ACN55, establishing the recA gene orientation (Fig. 4).
Plasmid integration and plasmid maintenance in mutant strains

A probe to the 3' region of trpD hybridized to fragments B1 and B2 of strain ACN30, suggesting duplication of this region. In the construction of ACN30, the use of linearized plasmid pBAC51 DNA should have led to the acquisition of drug resistance by allelic replacement of the chromosomal with the modified trpD locus. However, if the transforming DNA contained some circular rather than linear plasmid, homologous recombination could have integrated pBAC51 in the genome, resulting in chromosomal copies of both the wild-type and modified trpD alleles. A hybridization probe made to the vector portion of pBAC51 detected B1 of ACN30, indicating plasmid integration and demonstrating the trpGDC orientation (Fig. 4). Chromosomal integration of pBAC59 in the estR-disrupted ACN45 was similarly shown. A probe upstream of estR hybridized to both C1 and C2 of ACN45. A probe to pBAC59 vector sequences hybridized to fragment C1, allowing estR orientation (Fig. 4).

In ACN30 and ACN45, plasmid vector probes hybridized not only to the chromosomally integrated copies of plasmids, but also to the smallest DNA fragments generated by NotI cleavage of total DNA. These fragments corresponded in size to the plasmids used in ACN30 and ACN45 construction, pBAC51 and pBAC59, each of which has a single NotI recognition sequence. The intensities of these small DNA fragments suggested that they were present in more than one copy (Fig. 6). Similar results were found for strains ACN44, ACN33 and ACN67 (Fig. 6). Moreover, appropriate gene probes hybridized to these smaller fragments, suggesting that these five strains carried autonomously replicating plasmids.

**Locations of rrrn operons and IS1236 insertion sequences**

The restriction endonuclease I-CeuI recognizes a conserved 26 nucleotide sequence in bacterial rRNA operons (Liu et al., 1993). The presence of seven I-CeuI recognition sites indicated seven rRNA operons in ADP1. Consistent with the correlation between I-CeuI sites and rRNA operons, a ribosomal DNA probe (Dryden & Kaplan, 1990) hybridized only to NotI fragments A and D (data not shown).

Multiple copies of the insertion sequence IS1236 in ADP1 have been demonstrated (Gerischer et al., 1996). Their chromosomal locations were investigated here by Southern hybridization experiments with an IS1236-labelled DNA probe (Fig. 7). The relative intensities of hybridization signals suggested several copies of this insertion sequence on the wild-type NotI fragment C. Hybridization to Ascl-generated fragment 5, which is internal to fragment C, was also strong.

**DISCUSSION**

**Genome size and genetic organization**

This report presents the first physical and genetic map of the single, circular chromosome of *Acinetobacter* sp. strain ADP1. No evidence was found of endogenous plasmids. The genome size, 3.8±0.2 Mbp, is in a similar range to those of strains of *Rhodobacter*, *Salmonella*, *Escherichia*, *Bacillus* and *Pseudomonas* (Fonstein & Haselkorn, 1995). The number of ADP1 rRNA operons was found to be seven, the same as in *Pseudomonas putida* (Holloway et al., 1992) and *E. coli* (Ellwood & Nomura, 1982). Strain ADP1 grows quickly on rich medium, with doubling times of approximately 40 min, and the level of rrrn redundancy can provide the high ribosome levels needed for rapid growth (Condon et al., 1995). In a recent report, however, Stratz et al. (1996) demonstrated that in a BD413-derived strain of *Acinetobacter* sp., at least one rrrn operon could be inactivated without decreasing growth rates or causing detectable physiological changes. Their results were also consistent with the presence of seven *Acinetobacter* rrrn operons. In *E. coli*, the rrrn operons are clustered in one half of the chromosome, distributed around the origin of replication (Ellwood & Nomura, 1982). In ADP1, six of the seven rrrn operons were clustered in approximately one-third of the chromosome, but the position of these operons relative to the origin of replication was not investigated.

The relative positions were determined, however, of two supraoperonic gene clusters involved in dissimilation of aromatic compounds. Localization of the pcaD gene (map position 2555) determined the positions of the other known pca, qui and pob genes (not all shown on the map) which constitute an approximately 20 kbp
region involved in the degradation of aromatic com-

pounds via the protocatechuate branch of the β-keto-
adipate pathway (Gerischer & Ornston, 1995; Kloo
est et al., 1995). Moreover, the muck gene involved in cis,cis-

muconate metabolism (P. A. Williams, personal com-
munication), was found to be part of this cluster. Other
genes involved in cis,cis-muconate metabolism, the catBC1JFD

genes, are part of an approximately 20 kbp ben–cat

supraoperonic cluster encoding the catechol

branch of the β-ketoacidipate pathway (Ornston &

Neidle, 1991). Localization of catM, map position 2268,
established the relative positions of the other ben and cat

genes, some of which are shown on the map. A region of

280 kbp of DNA with unknown function separated the


Genes needed for aromatic compound degradation are

also clustered in both P. putida PPN (ATCC 12633) and

P. aeruginosa PAO (Holloway et al., 1990; Zhang et al.,

1993).

The clustering of these catabolic genes in Acinetobacter

sp. ADP1 is contrasted by the genomic separation of the

trp genes needed for tryptophan biosynthesis (Haspel

et al., 1991). In E. coli, there are five tryptophan genes,

trpEDCBA, which are contiguous in one operon, with the

trpD and trpC genes encoding bifunctional enzymes.

In Acinetobacter, the functions of the trpD-encoded

polypeptide are carried out by two proteins encoded by
distinct genes, trpD and trpG. Similarly, two genes, trpC

and trpF, encode the counterparts of the E. coli TrpC

polypeptide. As shown in these studies, the locations of the

trpE, trpFB, and trpGDC linkage groups are well

separated on the ADP1 chromosome. The location of

trpA was not determined. This arrangement is more

similar to that of pseudomonads than enteric bacteria.

In P. aeruginosa, there are at least four linkage groups,

trpE, trpGDC, trpF and trpBA, which are found in three

well-separated chromosomal locations (Haspel et al.,

1991; Holloway et al., 1990; Schmidt et al., 1996).

Mapping resolution

Genes were precisely localized by the introduction of

NotI recognition sites in the chromosome. Similar

mapping approaches have used transposons or plasmids
to introduce chromosomal restriction endonuclease

recognition sequences (reviewed by Fonstein &

Haselkorn, 1995). The ability to transform the wild-type

Acinetobacter strain with linear DNA or crude lysate

DNA in this investigation simplified the mutagenesis

procedure and facilitated the formation of strains with

multiple mutations. It was possible to determine the

transcriptional directions of individual genes and to

provide a high-resolution genetic map. In contrast, the

precision of gene localization by conventional

hybridization methods is limited by the size of the

smallest DNA fragment to which a probe hybridizes. In
general, the greater the number of recognition sites for

distinct endonucleases determined on a physical map,
the greater the genetic mapping precision by

hybridization. Extensive physical maps are often

constructed for the purpose of improving gene-mapping

resolution. With the methods used in these studies, a
detailed physical map was not required.

Southern hybridization techniques, however, can be

used to localize genes in ADP1 in those situations where

interposon mutagenesis is not feasible to introduce

specific DNA cleavage sites. Mapping precision can be

increased by the selected use and combination of the

engineered NotI sites in mutant strains. Cleavage of the

wild-type chromosome with NotI yields several large

DNA fragments that would not be useful as

hybridization targets for gene localization. Smaller

fragments can be generated by combining the interposon

and/or transposon insertions in different strains to be

present in a single strain. This method for cleaving large

fragments into several smaller fragments was demon-

strated in strains ACN55 and ACN65 used to orient the

recA and estA genes, respectively.

Hybridization methods might identify the ADP1 homo-

logues of genes isolated from other organisms. In vitro

mutagenesis approaches were unsuccessful with several

genes from A. calcoaceticus strains LMD79.41, NCIB

8250 and DSM 30007 (Gralton, 1996). Homology

between the DNA in the heterologous Acinetobacter

strains was apparently too low to introduce interposons

into the ADP1 chromosome. With Southern

hybridization techniques and reduced stringency

conditions, distantly related genetic homologues might be

identified.

Variability in the size of NotI-generated fragment C

During these investigations, a dramatic change was

noted in the size of the wild-type DNA fragment C

generated by chromosomal cleavage with NotI. Initially,

the fragment C was consistently found to be 653 kbp

(Fig. 1). This is the size of the corresponding fragment in

mutant strains ADP197, ADP212, ACN6.1, ACN16.1,

ACN27, ACN30, ACN44, ACN53 and ACN54 (Fig. 6).

In more recent TAFE analyses, however, the size of

fragment C appeared to be 550 kbp. This smaller-sized

fragment was designated C* (Fig. 6). Fragment C* was

also observed in the most recently constructed mutants

ACN66, ACN67, ACN74 and ACN79 (Fig. 6). These

changes may reflect a spontaneous deletion which

occurred in the laboratory version of the wild-type

strain. Use of this strain as the recipient in subsequent

transformation experiments would explain the presence

of C* in some mutants. The possibility that a deletion

occurred independently in different strains, however,
cannot be ruled out. No phenotypic differences have

been correlated with the presence of C*. Recent exami-
nation of NotI-digested DNA of strain ADP1 from L. N.

Ornston’s laboratory detected a 653 kbp fragment C

(data not shown).

Fragment C appeared to carry multiple copies of

insertion sequence IS1236 (Fig. 7). The possibility that

the deletion which occurred in fragment C is related to

interactions between different copies of IS1236 remains
to be investigated. In studies by Gerischer & Ornston

(1995), mutations leading to the inactivation of the
pcaHG genes, located on NotI fragment D, were shown to have been caused by the spontaneous insertion of IS1236 in this genetic region. As shown in the present study (Fig. 7), the wild-type strain does not have DNA homologous to IS1236 located on fragment D, indicating the ability of IS1236 to move to distant chromosomal locations.

Plasmids in Acinetobacter sp. ADP1

In several mutant strains, plasmid integration rather than allelic replacement occurred despite the use of linearized DNA in their construction. This suggests that the transforming DNA contained some circular rather than linearized DNA, a possibility consistent with the apparent maintenance of plasmids within some strains. The plasmids used in these studies all had ColE1-type replicons, and it has previously been assumed that these plasmids do not replicate in Acinetobacter (Hunger et al., 1990). Nevertheless, several lines of evidence indicated their autonomous replication in ADP1. The sizes of the smallest fragments observed in NotI-digested chromosomal DNA from strains ACN30, ACN44, ACN45, ACN53 and ACN67 were the same as those of the plasmids used to generate these mutants. In Southern hybridization experiments these same small fragments hybridized to probes of either plasmid vector sequences or the genetic region of the corresponding plasmid. Moreover, there were eight NotI cleavage fragments in each of these mutant strains, even though introduction of a novel chromosomal NotI recognition site would have generated only seven fragments.

The relative intensities of the smallest DNA fragments (Fig. 6) indicated several copies of each plasmid being maintained within the bacterial cells. Mutant strains maintaining plasmids had additional drug resistance conferred by the cloning vector, and in some cases plasmids could be cured from the Acinetobacter sp. strain (Gralton, 1996). This latter observation and the observation that the plasmids were present in higher copy number than chromosomal DNA fragments suggested that they were not generated by excision from the chromosome by homologous recombination. Consistent with this interpretation, plasmid size and linearity always corresponded to the disrupted allele being carried on the vector whereas excision would be expected to generate fragments with either the wild-type or the disrupted allele.

In the studies of Hunger et al. (1990) the ColE1-based plasmid pBR322 was not stably maintained in Acinetobacter strains. Factors which affect the maintenance and replication of plasmids in ADP1 remain to be defined. It is important to be aware of the possible autonomous replication of ColE1-based plasmids, especially in experiments where they have been used as ‘suicide’ plasmids to target single-copy genetic constructs for chromosomal integration. TAFE methods allow the presence of plasmids to be readily assessed. These mapping studies provide the foundation not only for future Acinetobacter gene localization and taxonomy studies, but also for investigations of plasmid maintenance and stability.

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