APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1993, p. 2807–2816
0099-2240/93/092807-10$02.00/0

Vol. 59, No. 9

Isolation, Characterization, and Sequence Analysis of Cryptic Plasmids from *Acinetobacter calcoaceticus* and Their Use in the Construction of *Escherichia coli* Shuttle Plasmids

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Received 4 January 1993/Accepted 12 June 1993

Three cryptic plasmids have been discovered in *Acinetobacter calcoaceticus* BD413. These three plasmids, designated pWM10 (7.4 kb), pWM11 (2.4 kb), and pWM12 (2.2 kb), exhibited extensive homology to one another, as shown by Southern blot hybridization and restriction site analysis data, and also hybridized with three plasmids having slightly different sizes detected in a second strain, *A. calcoaceticus* BD4. Plasmid pWM11 and a fragment of pWM10 were each subcloned into pUC19, yielding plasmids pWM4 and pWM6, respectively, and were used in a series of inter- and intraspecies transformation experiments. Both plasmids replicated as high-copy-number plasmids in *A. calcoaceticus* BD413, as well as in strains of *Escherichia coli*. However, when transformed into the oil-degrading strain *Acinetobacter lwoffii* RAG-1, both plasmids were maintained at low copy numbers. No modification of the plasmids was detected after repeated transfers between hosts. An analysis of a series of deletions demonstrated that (i) a 185-bp fragment of pWM11 was sufficient to permit replication of the shuttle plasmid in *A. calcoaceticus* BD413, (ii) the efficiency of transformation of *A. calcoaceticus* BD413 decreased according to the size of the deletion in the insert by up to 4 orders of magnitude, and (iii) the entire insert was required for transformation and replication in *A. lwoffii* RAG-1. The sequence of pWM11 contained several small (150- to 300-bp) open reading frames, none of which exhibited any homology to known DNA or protein sequences. In addition, a number of inverted and direct repeats, as well as six copies of the consensus sequence AAAAAAATA previously described for a cryptic plasmid from *A. lwoffii* (M. Hunger, R. Schmucker, V. Kishan, and W. Hillen, Gene 87:45–51, 1990), were detected. Cloning and expression of the alcohol dehydrogenase regulon from *A. lwoffii* RAG-1 were accomplished by using the *Acinetobacter* shuttle plasmid.

Members of the genus *Acinetobacter* are ubiquitous in nature, and these organisms are able to utilize a wide variety of carbon sources (41). Certain *Acinetobacter* species are well known for their clinical importance as opportunistic pathogens (16, 28, 42), while others have been considered to be of applied interest and potential industrial importance (11). One hydrocarbon-degrading species, *Acinetobacter lwoffii* RAG-1 (32), produces an amphipathic, polyanionic extracellular bioemulsifier, called emulsan, which stabilizes oil-in-water emulsions. Emulsan has been produced by fermentation at industrial levels, and the growth physiology of *A. lwoffii* RAG-1 has been extensively investigated (11).

Genetic studies of RAG-1 have been hampered by the lack of suitable systems for recombination and complementation in this organism. In contrast, *Acinetobacter calcoaceticus* BD4 and its miniencapsulated mutant *A. calcoaceticus* BD413 are naturally competent for transformation and provide a powerful tool for the identification and genetic analysis of other *Acinetobacter* systems (17, 18, 38, 39). Two alternative approaches to molecular analysis of *A. lwoffii* RAG-1 are (i) the cloning and expression of relevant genes from *A. lwoffii* RAG-1 in *Escherichia coli* (12) and (ii) the use of *A. calcoaceticus* BD4 and its minicapsular mutant *A. calcoaceticus* BD413 as recipients in transformation experiments by using putative homologous sequences from *A. lwoffii* RAG-1 cloned into *E. coli* plasmids (which cannot replicate in *Acinetobacter* strains) to generate mutations by homologous recombination (1a, 11, 23). While the latter approach can in principle be used to generate partial diploids in complementation analyses (23), it is not likely to be a simple procedure.

In this paper we describe the development of a shuttle plasmid which can be used for inter- and intraspecies transformations and gene expression in *E. coli* and *Acinetobacter* strains. The use of this plasmid in combination with electroporation for introduction of DNA should permit the development of a genetic system in the oil-degrading organism *A. lwoffii* RAG-1.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Two strains of *A. calcoaceticus*, the heavily encapsulated strain *A. calcoaceticus* BD4 and the minicapsular, tryptophan auxotroph *A. calcoaceticus* BD413 (18), were obtained from E. Juni. *A. lwoffii* RAG-1 (32) was obtained from our laboratory collection. Mutants of *A. lwoffii* RAG-1 deficient for growth on ethanol were isolated in this study (see below).

The following *E. coli* K-12 derivatives were used in this study: *E. coli* MM294 (26) (*pro thi* r^+ m^+ *recA*), *E. coli* TG-1 (10) [Δ(*lac-pro*) *supE A hsdD* F′ *tra* Δ*36*, $lacI^q$ *lacZ1ΔM15*], *E. coli* XL-1 Blue [*recA1 lac endA1 gyrA96 thi hsdR17 supE44 relA1* (F′ *proAB* $lacI^q$ *lacZΔM15*, Tn*10*)] (Stratagene), and *E. coli* MC1022 [*araD139*, Δ(*ara-leu*)*7697*, Δ(*lacZ*)*X74 M15 galU galK* Str] (4). *E. coli* RET-1 was

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derived from *E. coli* MM294 by introducing pRET-1, a plasmid that conferred growth on ethanol (12).

Acinetobacter strains were grown in liquid cultures either in minimal salt medium containing (per liter) 18.34 g of $K_2HPO_4 \cdot 3H_2O$ (Merck), 6 g of KH_2PO_4 (BDH), 0.2 g of $MgSO_4 \cdot 7H_2O$, and 4 g of $(NH_4)_2SO_4$ (Merck) and supplemented with 2% (vol/vol) ethanol as the sole source of carbon or in Luria broth (LB) (32). The minimal medium used for *A. calcoaceticus* BD413 and its derivates was supplemented with 50 μg of tryptophan per ml. *E. coli* strains were grown in minimal salt medium supplemented with 0.5% glucose and 1 μg of vitamin B1 per ml or in LB. *Acinetobacter* strains were grown at 30°C, while *E. coli* strains were incubated at 37°C. Sodium acetate (2%, vol/vol) triacetin (5 mM) (31), and hexadecane (1% [vol/vol] in liquid cultures or a vapor from a filter containing 0.1 ml of hexadecane that was placed in the lid of a petri dish) were used as alternative carbon sources. Unless indicated otherwise, the concentrations of antibiotics used were 50 μg/ml for ampicillin and kanamycin and 12.5 μg/ml for tetracycline.

Plasmids. Plasmids pUC19 (Bethesda Research Laboratories), pUC4-k (Pharmacia), and pBluescriptM13-KS (Stratagene) were used for cloning, subcloning, and sequencing of *Acinetobacter* DNA. Plasmid pRET-1 (30) was obtained from R. Petter (Department of Biophysics, Weizmann Institute of Science, Rehovoth, Israel); it consisted of a 5.6-kb insert from *A. lwoffii* DNA cloned into the *tet* resistance gene of pBR322.

Zymogram analysis of ADH. Alcohol dehydrogenase (ADH) activity was determined in crude cell extracts from cultures grown in the presence of ethanol by using a method similar to the one described by Singer and Finnerty (36). Cells from overnight cultures (50 ml) of *Acinetobacter* strains grown either in minimal salts medium supplemented with ethanol or in LB supplemented with ethanol and of *E. coli* strains grown in minimal salts medium supplemented with ethanol, vitamin B1, and either LB or glycerol (0.2%, vol/vol) were harvested by centrifugation and washed once in 10 mM Tris buffer (pH 7.8), and each resulting pellet was resuspended in a solution containing 1 ml of 10 mM Tris (pH 7.8) and 2 ml of lysis solution (6 mg of lysozyme per ml, 0.1 ml of 6% Triton X-100 per ml, and 6 μg of DNase per ml in 10 mM Tris [pH 7.8]). The mixture was incubated for 30 min at 25°C or until complete lysis was obtained. The cell debris was pelleted by centrifugation, and the supernatant was retained for zymogram analysis. Between 30 and 50 μg of protein from the extract was subjected to electrophoresis in a nondenaturing 7.5% acrylamide gel by using the 25 mM Tris–glycine buffer system (21). Samples were loaded onto two gels in order to determine the NAD^+- and $NADP^+$-dependent ADH activities. After separation was completed, the gels were soaked in 100 ml of 50 mM Tris buffer (pH 8.8) containing 40 mg of Nitro Blue Tetrazolium (Sigma), 8 mg of phenazine methosulfate (Sigma), 40 mg of either NAD^+ or $NADP^+$, and 1 ml of ethanol. The gels were kept in the dark at room temperature for 30 min or until dark blue bands corresponding to sites of ADH activity appeared. Protein was determined by using the Bio-Rad reagent, the method of Bradford (3), and bovine serum albumin as the standard.

Transformation of *E. coli* and *Acinetobacter* strains. *E. coli* strains were transformed by standard procedures (6, 24). Competent cells were stored at −70°C. The naturally competent strain *A. calcoaceticus* BD413 (17) was transformed by using the protocol described by Singer and coworkers (37).

For transformation by electroporation, cells were prepared in the following manner. An overnight culture was diluted 1:150 into 1 liter of LB, and the preparation was shaken at 37°C for 2 to 3 hours until the A_{600} was between 0.5 and 1.0. The culture was chilled on ice, centrifuged for 20 min at 8,000 rpm at 4°C in a GSA rotor, and resuspended in 100 ml of ice-cold sterile double-distilled water. After three washes with sterile ice-cold water, the cells were resuspended in 20 ml of ice-cold 10% glycerol, centrifuged again, and resuspended in 2 ml of ice-cold 10% glycerol. The resulting cells were divided into 200-μl aliquots and stored frozen at −70°C. For electroporation the cells were allowed to thaw on ice, transforming DNA was added (not more than 1 μl of DNA), and the mixture was kept on ice for 1 min. The cells were transferred into a Bio-Rad Gene Pulser cuvette with a 0.2-cm pathlength. Electroporation was performed with a Bio-Rad Gene Pulser apparatus with a constant capacitance of 25 μF, a constant resistance of 200 Ω, and a time constant of 4.5 ms. For *Acinetobacter* cells 2,000 V was applied, while 2,500 V was used for *E. coli* MC1022 cells; these settings resulted in electrical fields of 10,000 and 12,000 V/cm, respectively. After electroporation, the cells were suspended in 1 ml of prewarmed LB and incubated for 2 h at 37°C (*E. coli*) or for 4 h at 30°C (*Acinetobacter* strains) to allow for phenotypic expression prior to plating on selective medium.

Preparation of total genomic DNA. Total genomic DNA was isolated from *Acinetobacter* strains by the method described by Hopwood et al. (14). This procedure yielded high-molecular-weight genomic DNA (length, >30 kb).

Plasmid DNA isolation. Several methods were used to isolate plasmid DNA form *E. coli* or *Acinetobacter* strains. The boiling method (13) was used for rapid screening of recombinant plasmids. If DNA of higher purity was required, a modified alkaline lysis method was used (2, 24). DNA prepared by the latter procedure was purified on an Elutip minicolumn (Schleicher & Schuell) and was found to be suitable for double-stranded DNA sequencing.

Isolation of ethanol-deficient mutants of *A. lwoffii* RAG-1. Cultures of *A. lwoffii* RAG-1 were concentrated and plated ($\sim 10^{10}$ cells per plate) on LB plates containing 20 to 50 mM allyl alcohol (22). Cells possessing active ADH activity metabolize the suicide substrate allyl alcohol and the cells are killed, while mutants lacking ADH activity are not killed.

Common techniques used in molecular biology. Common techniques used in molecular biology, including agarose gel electrophoresis, elution of fragments, DNA modification, colony hybridization, and Southern blot analysis were performed as described by Maniatis et al. (24). When blots were used for subsequent hybridizations with different probes, the filters were stripped by washing them twice for 10 min at room temperature in 0.1 M NaOH–10 mM EDTA and then twice for 15 min at room temperature in 2× SSC–0.1% sodium dodecyl sulfate. Stripped filters were exposed overnight to confirm the removal of all label before hybridization was attempted with a second probe.

Double-stranded DNA fragments cloned in either pUC19 or pBluescriptM13-KS were sequenced by using the chain termination DNA sequencing method (34) and Sequenase, version 2.0 (USB). Single-stranded DNA was obtained from pBluescript vectors in combination with *E. coli* XL-1 Blue. Phage DNA was isolated by standard procedures (24) or as described in the Bluescript manual (Stratagene).

DNA sequence analysis. The DNA Strider program was used to determine restriction sites and to find open reading frames. Direct and inverted repeats were detected by using the Beckmann MicroGenie program. The GenBank library

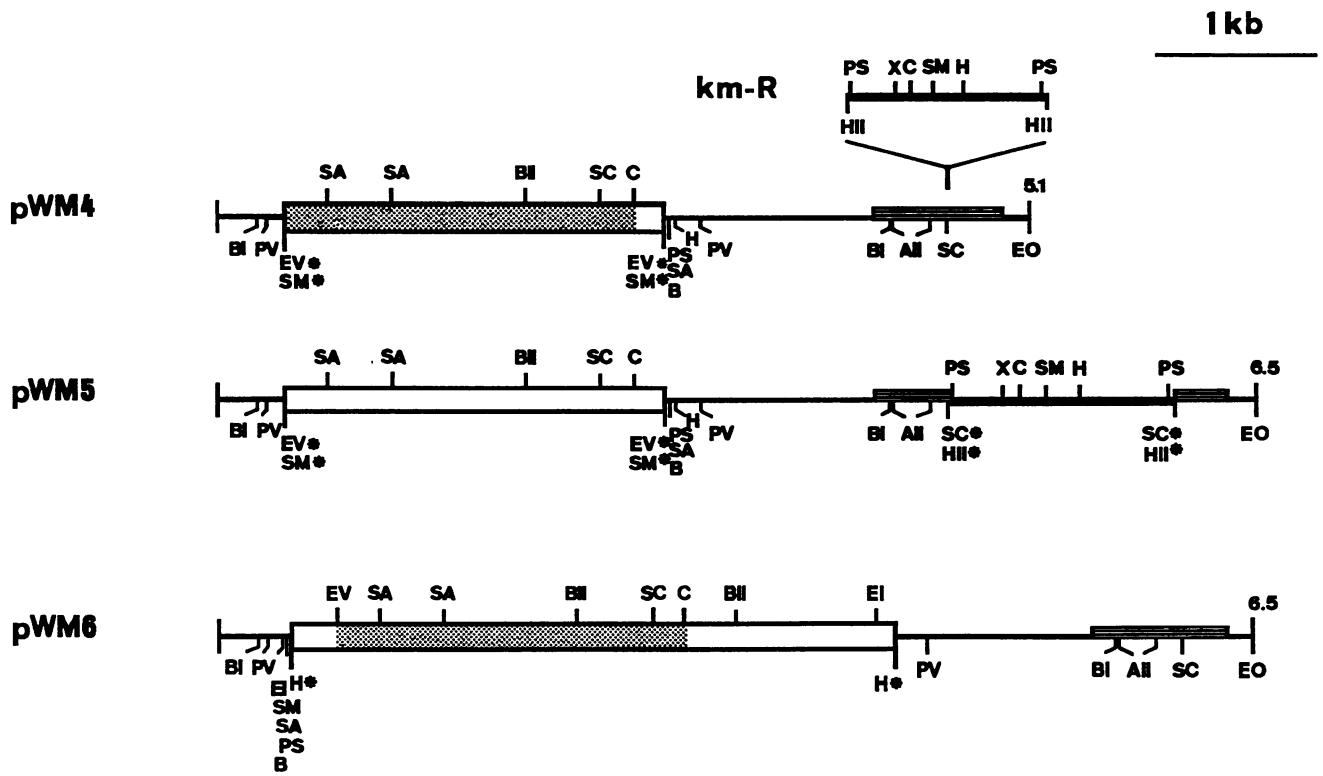


FIG. 1. Partial restriction maps of pWM4, pWM5, and pWM6. Inserts of *A. calcoaceticus* WM34 plasmid DNA cloned into pUC19 are indicated by boxes. The shaded areas stretching from the *Eco*RV site to the *Cla*I site in pWM4 and pWM6 are areas in which the restriction sites for the two cloned fragments are identical (see text for details). Plasmid pWM5 is a derivative of pWM4 in which the kanamycin resistance cassette from pUC4-K was inserted into the *bla* gene as shown. No restriction sites were found for *Bst*EII and *Xho*I. Abbreviations: AII, *Ava*II; B, *Bam*HI; BI, *Bgl*I; BII, *Bgl*II; C, *Cla*I; EO, *Eco*O109; EI, *Eco*RI; EV, *Eco*RV; HII, *Hin*cII; H, *Hin*dIII; PS, *Pst*I; PV, *Pvu*II; SA, *Sal*I; SC, *Sca*I; SM, *Sma*I; X, *Xho*I. The asterisks indicate sites lost during cloning.

and the EMBL library (in November 1992) were searched for sequence homologies by using the GCG program package (Genetics Computer Group, University of Wisconsin Biotechnology Center, Madison) (8).

Nucleotide sequence accession number. The GenBank accession number for the DNA sequence of plasmid pWM11 is L08076.

RESULTS

Detection of cryptic plasmids in *A. calcoaceticus* BD413. In a series of experiments to be described elsewhere, specific mutations in *A. calcoaceticus* BD413 were generated by transformation with genomic libraries prepared from other *Acinetobacter* strains. These libraries were constructed in *E. coli* plasmids (pUC) that do not replicate in *Acinetobacter* strains. When the vector was used as a probe, Southern blot analysis revealed that the plasmids had integrated into the chromosomes of the transformants of *A. calcoaceticus* BD413 (26a). During the screening of parental strain *A. calcoaceticus* BD413 and 120 mutants, only one transformant, designated *A. calcoaceticus* WM34, was found to contain five ethidium bromide-stained bands in undigested plasmid preparations. All of the bands were sensitive to DNase treatment but resistant to RNase digestion. A series of restriction digestion experiments were performed, and the banding pattern obtained was compared with fragments expected from the cloning vector used in transformations of *A. calcoaceticus* BD413. No vector fragments were identified among the *A. calcoaceticus* WM34 digests. Moreover, in Southern blot experiments in which the α-^{32}P-labelled vector was used as the probe, no homology was detected to the extrachromosomal elements even when the hybridization experiments and washing were performed under the most mild conditions.

Exhaustive digestion of this extrachromosomal DNA with different restriction enzymes yielded a pattern of fragments in which some of the bands of undigested DNA disappeared, while others remained unaltered. An analysis of the restriction endonuclease profiles indicated that *A. calcoaceticus* WM34 contained three plasmids, which were designated pWM10 (7.4 kb), pWM11 (2.4 kb), and pWM12 (2.2 kb).

A. calcoaceticus WM34 contained enough extrachromosomal DNA in standard plasmid preparations that it could be seen in ethidium bromide-stained gels. Corresponding extrachromosomal DNA bands from *A. calcoaceticus* BD4 and BD413 were subsequently detected only in undigested total DNA preparations. In agreement with previous results (33), no extrachromosomal DNA was observed in *A. lwoffii* RAG-1 (data not shown).

Subcloning of plasmid DNA from *A. calcoaceticus* WM34 into pUC19. Several attempts were made to isolate the three plasmids of both *A. calcoaceticus* BD413 and WM34 individually in order to further investigate their properties (Fig. 1). The lack of a selectable marker and specific origin of replication precluded direct transformation into *E. coli*. A standard plasmid preparation from *A. calcoaceticus* WM34 was digested with enzymes known to cleave only one or two

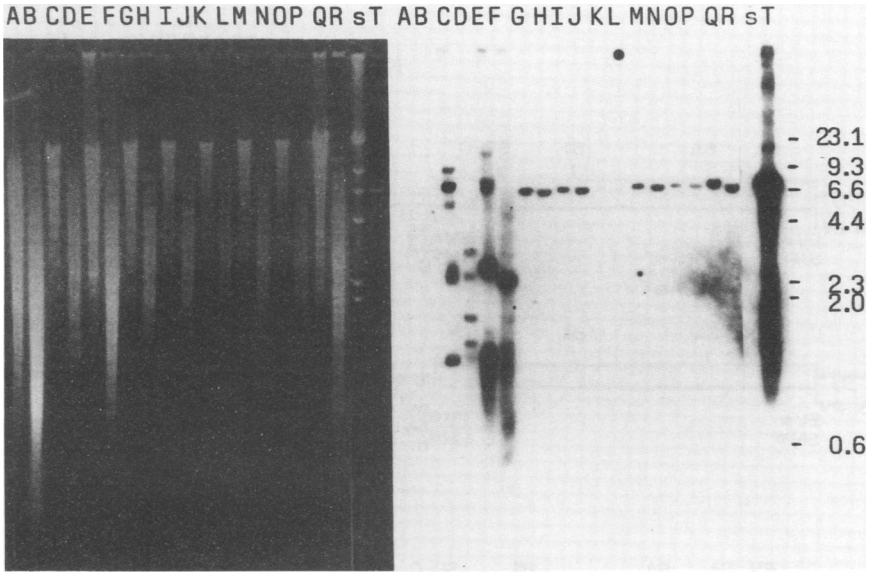


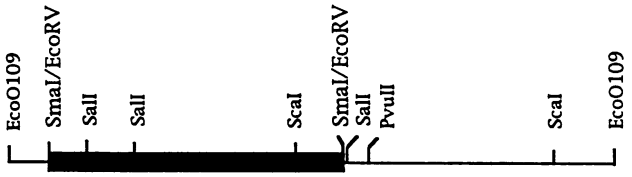
FIG. 2. Agarose gel and Southern blot analysis of different *Acinetobacter* strains and six *A. lwoffii* RAG-1 transformants hybridized with pWM5. Total DNAs of *A. lwoffii* RAG-1 (lanes A and B), *A. calcoaceticus* BD4 (lanes C and D), *A. calcoaceticus* BD413 (lanes E and F), and the six *A. lwoffii* RAG-1(pWM5) transformants (lanes G and H, I and J, K and L, M and N, O and P, and Q and R) were digested with *Bcl*I (lanes A, C, E, G, I, K, M, O, and Q) or *Xba*I (lanes B, D, F, H, J, L, N, P, and R). *Hin*dIII-digested lambda DNA was used as the size marker (lanes s). The sizes of the fragments (in kilobases) are indicated on the right. *Bam*HI-digested pWM5 (lanes T) was used as a control for the hybridization.

of the plasmids (data not shown) and ligated into pUC19. Two recombinant plasmids were chosen for further analysis. Plasmid pWM4 was the result of the ligation of *Sma*I-digested pUC19 and an *Eco*RV digest of the plasmids. *Eco*RV cleaved the 2.4-kb plasmid pWM11 as well as the 7.4-kb plasmid pWM10 once. As determined by the size of pWM4, plasmid pWM11 was ligated into pUC19. Plasmid pWM6 was obtained after digestion of pWM10 with *Hin*dIII (cleaved twice) and ligation of the 3.9-kb fragment into pUC19 digested with the same enzyme. Restriction maps for some 20 restriction endonucleases for both recombinant plasmids were determined (Fig. 1). Of interest was the finding that a 2.1-kb internal fragment of the insert of pWM6, extending from the *Eco*RV site to the *Cla*I site, had restriction sites identical to those of the insert of pWM4 (Fig. 1, shaded areas of pWM4 and pWM6).

Distribution of *A. calcoaceticus* BD413 plasmids. Plasmids pWM4 and pWM6 were used as probes in a Southern blot analysis of total DNAs isolated from several *Acinetobacter* and *E. coli* strains. Only extrachromosomal DNAs prepared from (i) *A. calcoaceticus* BD413, (ii) transformants of *A. calcoaceticus* BD413, and (iii) *A. calcoaceticus* BD4 exhibited homology to the probes (Fig. 2, lanes A through F). It is interesting that when blots hybridized with pWM4 were washed and rehybridized with pWM6, identical hybridization patterns were obtained, indicating that cryptic plasmids pWM10 and pWM11 of *A. calcoaceticus* BD413 were highly homologous, which was consistent with the restriction analysis results described above.

Transformation of *E. coli* and *A. calcoaceticus* BD413 with pWM4 and pWM6. *A. calcoaceticus* BD413 was readily transformed with pWM4 and pWM6 to yield ampicillin-resistant transformants at a frequency of 10^5 cells per μg of DNA. Furthermore, when plasmid DNAs isolated from *A. calcoaceticus* BD413 transformants were retransformed into *E. coli* MM294, similar transformation efficiencies were obtained. After each transformation, plasmid DNA was isolated, and the size and presence of restriction sites in the plasmids were examined. No rearrangements or modifications of either pWM4 or pWM6 were observed after two reciprocal transfers between *A. calcoaceticus* BD413 and *E. coli* MM294. It is interesting that the amount of shuttle plasmid recovered from either *A. calcoaceticus* or *E. coli* was in the order of 1 μg of DNA per ml of culture (about 10^9 cells). In the case of *A. lwoffii* RAG-1 transformants (10^9 cells), nanogram quantities of shuttle plasmid DNA were recovered only in total DNA preparations which were subsequently used in interspecies transformations.

Deletions of pWM4 and their effect on transformation in *Acinetobacter* strains. Three *Sal*I sites were detected in pWM4; two of these were localized to the inserted DNA from pWM11, while the third site was in the polylinker of pUC19 (Fig. 3). By using *Sal*I, different deletions of the cloned fragment were generated. After partial digestion and self-ligation three plasmids were isolated: pWM41 lacking the 0.6-kb *Sal*I fragment, pWM42 lacking the 1.7-kb fragment, and pWM43 lacking both fragments (2.3 kb), leaving only 185 bp of the original insert. The three plasmids were used to transform *A. calcoaceticus* BD413, *A. lwoffii* RAG-1, and *E. coli* MM294. The efficiencies of transformation (expressed as numbers of stable ampicillin-resistant transformants per microgram of DNA) were compared with the efficiencies of transformation of pWM4 (Fig. 3). As expected, the deletion of segments from the insert had little effect on transformation in *E. coli*. In contrast, the efficiency of transformation in *A. calcoaceticus* BD413 was reduced by almost 1 order of magnitude when the 0.6-kb fragment was deleted (pWM41). In the case of pWM42, which was missing the 1.7-kb fragment, the efficiency of transformation was reduced by an additional 30-fold. When both fragments were deleted (pWM43), transformation still occurred, but the efficiency of transformation was reduced by more than 4



plasmid	BD413	RAG-1	MM294
	Transformants per μg of DNA		
pWM4	6×10^5	5×10^5	1×10^5
pWM41	9×10^4	0	2×10^5
pWM42	3×10^3	–	2×10^5
pWM43	4×10^1	0	3×10^6

FIG. 3. *SalI* deletions of pWM4 and their effect on transformation of *A. calcoaceticus* BD413, *A. lwoffii* RAG-1, and *E. coli* MM294. The deletions of pWM41, pWM42, and pWM43 were 0.6, 1.7, and 2.3 kb long, respectively. The gaps in the plasmid maps indicate the deleted regions. The solid boxes represent the insert of pWM11 in pUC19 (line). Efficiencies of transformation (expressed as number of transformants per microgram of DNA) are shown on the right. For strain RAG-1 transformation was by electroporation.

orders of magnitude compared with the original plasmid, pWM4. Furthermore, only pWM4 was effective in transforming *A. lwoffii* RAG-1 (using electroporation) (see below). When the 0.6- and 1.7-kb *SalI* fragments (both lacking the 185-bp region) were subcloned into pUC19 and used for transformation, no stable transformants of either *A. lwoffii* RAG-1 or *A. calcoaceticus* BD413 were obtained (data not shown). Since *A. lwoffii* RAG-1 was somewhat resistant to ampicillin, a modification of pWM4 was introduced for subsequent transformation experiments in this strain. The kanamycin resistance gene, isolated from plasmid pUC4-K, was inserted into the *ScaI* site of the *bla* gene of pWM4. The resulting plasmid, pWM5, was 6.5 kb long (Fig. 1). Transformation with intact pWM5 and transformation with derivatives of pWM5 containing the corresponding deletions were similar in all respects to the transformation described in Fig. 3. Finally, *SalI* fragments (0.6 and 1.7 kb, the results of internal deletions) were subcloned either into pUC19 or into pBluescript and used in transformation experiments. Transformants were obtained only when strains of *E. coli* or *A. calcoaceticus* BD413 was used as the recipient. *A. lwoffii* RAG-1 itself could not be transformed with these subcloned fragments (data not shown).

Transformation of *A. lwoffii* RAG-1 with the shuttle plasmids. Although attempts to transform *A. lwoffii* RAG-1 with pWM4 and pWM5 by using a variety of conventional protocols for preparation of competent cells were not successful, transformants of *A. lwoffii* RAG-1 were obtained by using electroporation. When pWM5, which was isolated after transformation from either *A. calcoaceticus* BD413 or *E. coli* MM294, was used as the transforming DNA, efficiencies of about 10^5 transformants of *A. lwoffii* RAG-1 per μg of DNA were obtained. However, in contrast to *A. calcoaceticus* BD413 or *E. coli* MM294, in which significant quantities of pWM5 could be extracted from the transformants, essentially no plasmid DNA was detected in the transformants of *A. lwoffii* RAG-1. In order to differentiate between the possibility that pWM5 had integrated into the chromosome and the possibility that pWM5 had replicated independently but at a much lower copy number, total DNAs were extracted from six *A. lwoffii* RAG-1 transformants and *A. lwoffii* RAG-1 itself, as well as *A. calcoaceticus* BD4 and *A. calcoaceticus* BD413. These preparations were digested with *BclI* or *XbaI*, each of which cleaved pWM5 once. The resulting DNA samples were then used in Southern blots by using labelled pWM5 as the probe (Fig. 2). As expected, no homology was found between *A. lwoffii* RAG-1 DNA and the labelled plasmid. However, each of the six transformants yielded a 6.5-kb band which was identical to the linearized form of pWM5. Similarly, digestion of the total DNAs from the transformants with *BglI*, *ClaI*, or *Hin*dIII, each of which cleaved pWM5 twice, yielded fragments of the expected size, as if the plasmid were not integrated into the chromosome. Our results support the conclusion that pWM5 replicated independently in *A. lwoffii* RAG-1. Consistent with this was the finding that total DNAs extracted from the six *A. lwoffii* RAG-1 transformants could be used to transform either *A. calcoaceticus* BD413 or *E. coli* MM294 to kanamycin resistance with high efficiency. The kanamycin-resistant transformants yielded high levels of pWM5 which appeared to be unmodified, as judged by restriction analysis (data not shown). As expected, even in the absence of transformation, total DNAs from both *A. calcoaceticus* BD4 and *A. calcoaceticus* BD413 exhibited homology to pWM5 because of the presence in these strains of endogenous plasmids.

Nucleotide sequence and sequence analysis of cryptic plas-

mid pWM11. The nucleotide sequence of the 2.4-kb plasmid pWM11 was determined after *Sal*I fragments of pWM4 were first subcloned into either pUC19 or pBluescriptM13-KS. The 1.7-kb *Sal*I fragment was subcloned into the *Sal*I site of pBluescript. Deletions were generated by using (i) the *Cla*I site in the cloned fragment and the *Cla*I site in the polylinker of pBluescript M13-KS, (ii) the *Eco*RV site in the cloned fragment and the *Sma*I site in the polylinker, or (iii) the *Bgl*II site in the fragment and the *Bam*HI site in the polylinker. After self-ligation these deletions were used for double-stranded sequencing. The sequence is shown in Fig. 4. The computer analysis of the sequence included a search for open reading frames, repetitive sequences, secondary structure, and homology to other known sequences and origins of replication (see Methods and Materials).

While a number of translational start and stop sites were identified within the sequence, none of the open reading frames were more than 300 bp long. Five of the largest open reading frames were selected and analyzed for DNA and protein sequence homology (see Materials and Methods). No sequence homologies were detected either to structural genes, proteins, or the known origins of replication listed in the protein and DNA sequence data banks. The G+C content of pWM11 was 38 mol%, which is typical for *Acinetobacter* DNA.

As indicated in Fig. 4 (arrows), the sequence contained a number of inverted repeats. Direct repeats (data not shown) were also detected. In addition, a sequence which differed by no more than 2 bp from a 9-bp consensus motif (15), AAAAAATAT, was found repeated four times (258 to 267, 324 to 333, 511 to 520, and 534 to 543 bp) within a region of 295 nucleotides. Two additional AAAAAATAT sequences at 786 to 777 and 1767 to 1758 bp were oriented in the opposite direction.

Cloning of an ADH gene in pWM4. Previous studies performed in our laboratory led to the development of recombinant plasmid pRET-1 (11, 30), which confers on *E. coli* MM294 the ability to utilize ethanol as a sole source of carbon and energy because of expression of an ADH regulon from *A. lwoffii* RAG-1. In order to test the potential utility of shuttle plasmid pWM4 as a cloning vector, the genes coding for the *A. lwoffii* RAG-1 ADH activity were transferred from pRET-1 into pWM4. A *Cla*I fragment of pRET-1 was isolated which contained the tetracycline promoter from which ADH activity is transcribed in pRET-1 together with 4.5 kb of the 5.2-kb insert encoding ADH activity. This *Cla*I fragment of pRET-1 was subcloned into pWM4 by using either the *Acc*I site (pWM402) or the *Cla*I site (pWM412) (Fig. 5). The *Cla*I site is located in the pWM11 portion of the shuttle plasmid, whereas the *Acc*I site is located in the polylinker of pUC19 present in pWM4. Following transformation of *E. coli* MM294, the cells were spread on plates containing ampicillin and ethanol as the sole source of carbon and energy. After 5 days, ethanol-positive transformants were identified, and plasmids prepared from colonies were isolated from both ligation preparations. In both cases, the insert encoding the ADH coding region was cloned in the same orientation into pWM4 (Fig. 5). According to the zymogram data shown in Fig. 5, the same ADH activity was detected in all samples, even though the subcloned fragment was approximately 700 bp smaller than the original insert in pRET-1. As described previously for pRET-1 (11), three bands of $NADP^+$-dependent ADH activity were detected (Fig. 5), the slowest of which can also utilize NAD^+. All ADH activities associated with the subclones migrated with the same electrophoretic mobility under nondenaturing conditions as *E. coli* RET-1 activities. Band 1 appeared to be more active in *E. coli* RET-1 than in the strains containing the shuttle plasmid, while the strains carrying the shuttle plasmids exhibited elevated activities associated with band 2. Band 3 and the NAD^+-dependent activities were similar in extracts from all of the strains.

In order to examine whether the subcloned ADH genes were expressed in *Acinetobacter* strains, two mutants of *A. lwoffii* RAG-1, *A. lwoffii* WA1 and YB (both allyl alcohol-resistant strains unable to grow on ethanol), were transformed with either pWM402 or pWM412. About 2×10^3 ethanol-positive, ampicillin-resistant transformants per μg of DNA were obtained.

DISCUSSION

Most natural *Acinetobacter* isolates appear to contain plasmids which are either cryptic or encode resistance to antibiotics or heavy metals (41). In addition, a variety of metabolic and degradative plasmids have also been described. Rusansky et al. (33) described an *Acinetobacter* isolate, *A. lwoffii* RA57, which contained three plasmids, one of which appeared to be required for growth and emulsification of crude oil in liquid culture. The plasmid in this case was apparently required for mediating the interaction of the organism at the oil-water interface.

This study is the first report in which plasmids in *A. calcoaceticus* BD4 and BD413 are described. These strains have proven to be very important in the study of *Acinetobacter* genetics, primarily because of their capacity for highly efficient transformation by DNA from any *Acinetobacter* strain (40).

Our findings appear at first glance to be in conflict with the generally accepted notion that *A. calcoaceticus* BD413 is a plasmid-free strain (35). However, it should be noted that only one transformant of *A. calcoaceticus* BD413, *A. calcoaceticus* WM34, yielded sufficient extrachromosomal DNA in standard plasmid preparations to be detected by ethidium bromide staining on agarose gels. In all other cases, the plasmids were detected only in total DNA preparations, which generally yielded higher amounts of both chromosomal and plasmid DNAs and which permitted the detection of nanogram quantities of DNA in Southern blot experiments. The plasmid profiles of *A. calcoaceticus* WM34 and BD413 were identical, indicating that strain WM34 did not carry new extrachromosomal elements. While the plasmid profiles of *A. calcoaceticus* BD413 and WM34 were identical, the migration of *A. calcoaceticus* BD4 plasmids was slightly different. However, Southern hybridization in which the *A. calcoaceticus* BD413 plasmids were used as probes revealed a high level of DNA homology.

The question remains as to how *A. calcoaceticus* WM34, a transformant of *A. calcoaceticus* BD413, permits the replication of its plasmids to a higher copy number than *A. calcoaceticus* BD413 itself. One explanation consistent with these results is that *A. calcoaceticus* WM34 is a mutant in which the negative control of plasmid copy number has been relieved. The initial transformation might have resulted in the disruption of a gene coding for a product(s) which exerts negative control on replication (5). A second question which arises is how the apparent copy number of the shuttle plasmid in *A. calcoaceticus* BD413 is higher than the copy number of the cryptic plasmids. This might have resulted from the efficient fusion of a high-copy-number plasmid (pUC) with a fragment of *Acinetobacter* plasmid DNA necessary for maintenance. This is consistent with the

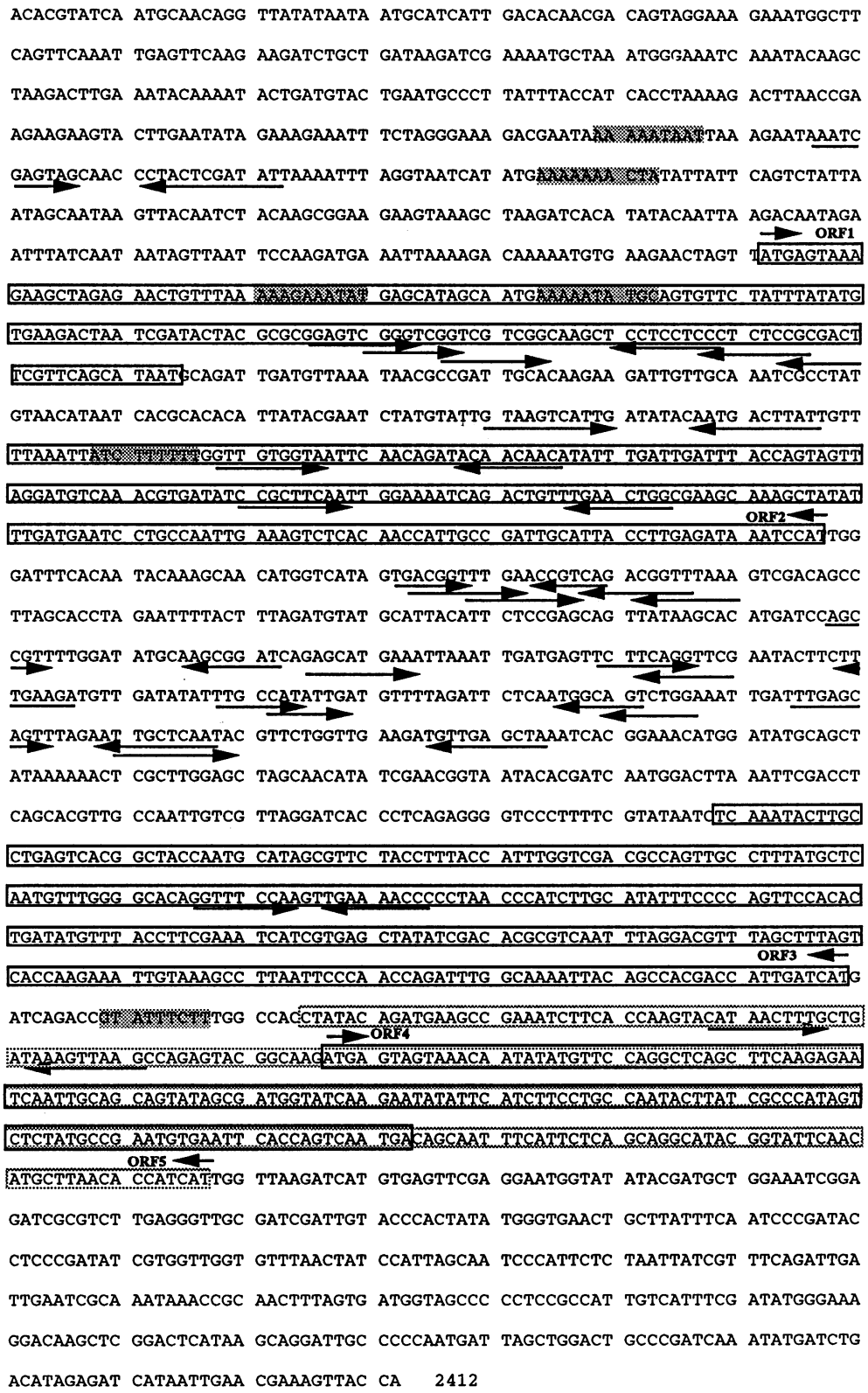


FIG. 4. Nucleotide sequence of the cryptic *A. calcoaceticus* BD413 plasmid pWM11. The arrows indicate the positions and extents of some of the inverted repeats found by computer analysis. The boxes around sequences designated ORF1 through ORF5 and the arrows above them indicate the positions and directions of the five largest open reading frames. The sequences in shaded areas are the 9-bp consensus sequence AAAAAATAT (see text).

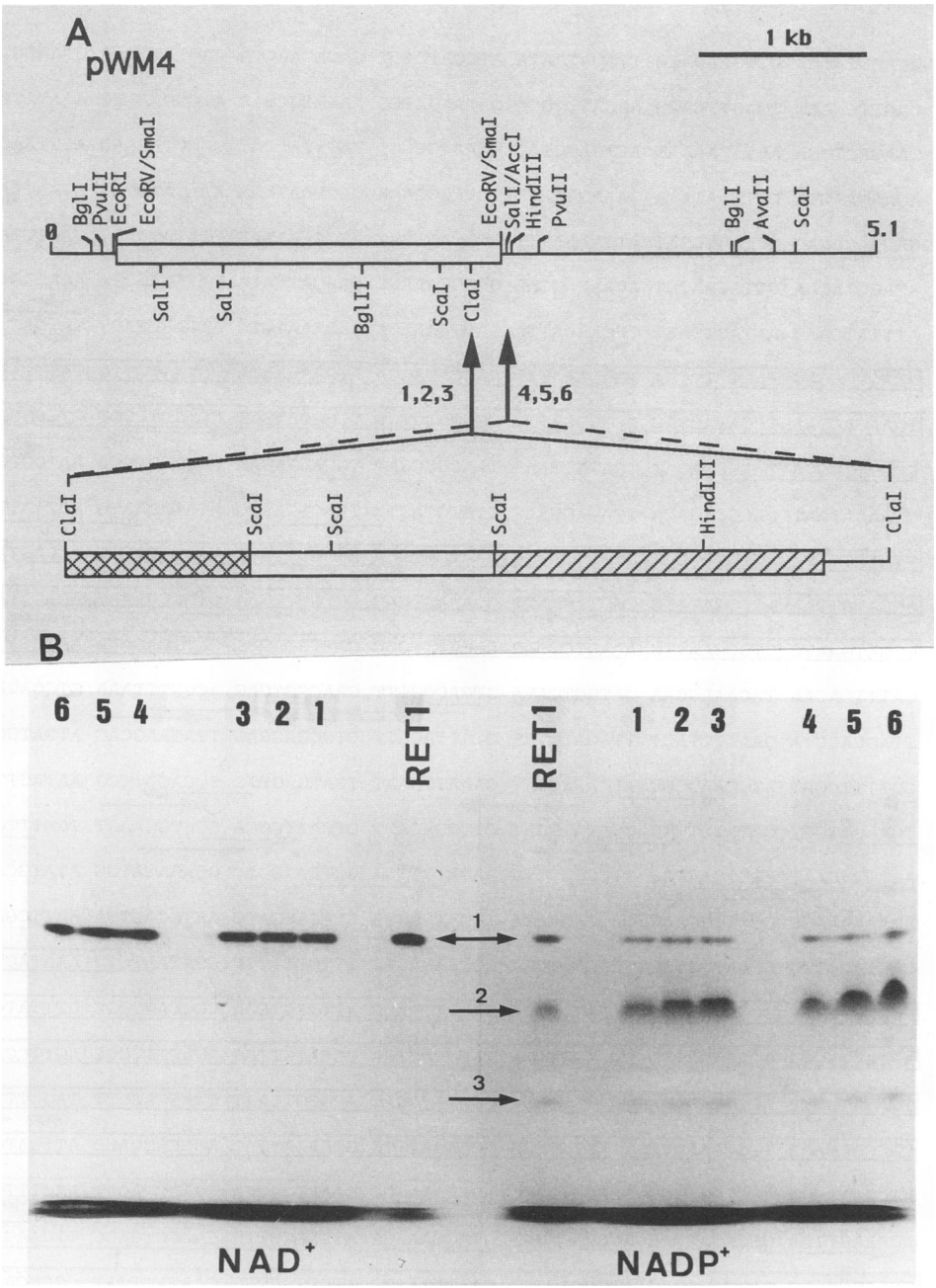


FIG. 5. (A) Recombinant shuttle plasmids pWM402 and pWM412. *Acc*I and *Cla*I digests of pWM4 were ligated with the *Cla*I fragment of pRET-1 that codes for ADH activity to yield pWM402 (clones 1 through 3) and pWM412 (clones 4 through 6), respectively. The region extending to the right of the last *Sca*I site to the end of the fragment (diagonally hatched) codes in *E. coli* for the NAD^+-dependent ADH activity. The left portion of the insert from *Cla*I to the first *Sca*I site (cross-hatched) codes for the NAD^+-dependent and $NADP^+$-dependent ADH activities (30). Both recombinant plasmids confer on *E. coli* MM294 the ability to grow on ethanol as a sole source of carbon and energy. (B) Zymograms of ADH activities from strains of *E. coli* MM294 transformed with pWM402 (lanes 1 through 3) and pWM412 (lanes 4 through 6) and strain RET-1. The dark line at the bottom is the migration front of the sample dye. The bands of ADH activity indicated by the arrows correspond to the typical isoenzyme pattern established for ADH from *A. lwoffii* RAG-1 (30).

finding that the transformation efficiency into *Acinetobacter* strains correlated with the size of the fragment fused to pUC.

It is difficult to understand how all three plasmids (pWM10, pWM11, and pWM12) are stably maintained in the same cell. Given their extensive homology, it seems unlikely that these plasmids belong to different incompatibility groups (7, 29), although this explanation cannot be completely ruled out. These plasmids do not appear to be multimeric forms of a single species, since several enzymes cleaved only one or two of the plasmids (e.g., *Eco*RI cleaved only pWM10, and *Xho*I cleaved pWM12 but not the other plasmids, while *Cla*I cleaved both pWM10 and pWM11 but

not pWM12). One possibility is that the plasmids are strongly associated with the chromosome (19, 20). It has been suggested that approximately 24% of the ColE1 plasmid population in *E. coli* is trapped in folded chromosomes and that these plasmids are in equilibrium between their associated and free forms (19, 20). This kind of physical attachment may account for the fact that the presence of plasmids was established only in total DNA preparations. This could also explain the fact that extrachromosomal shuttle plasmid DNA escaped detection in the *A. lwoffii* RAG-1 transformants.

A sequence analysis of pWM11 revealed a large number of translational start and stop codons, although only a few short open reading frames (size, 150 to 300 bp) were detected. In addition, a high number of direct and inverted repeats, several of which were found clustered (Fig. 4), were also observed. It is not clear whether translation of any of the plasmid sequences is necessary for maximal replication in the *Acinetobacter* strains. It was of interest in this regard that deleting fragments from the pWM11 portion of pWM4 reduced the efficiency of transformation of *A. calcoaceticus* BD413 in proportion to the size of the deletion. The minimal coding region of a plasmid whose autonomous replication was dependent on a Rep-like protein was found to be about 1 kb long (9). Nevertheless, a 185-bp cloned fragment was sufficient to allow replication of the shuttle plasmid in *A. calcoaceticus* BD413. A different cryptic plasmid has been reported in a different strain of *A. lwoffii* (15). In this case there also appeared to be no requirement for expression of a plasmid-mediated function in order to obtain plasmid maintenance and replication. Hunger et al. (15) identified the 9-bp motif AAAAAATAT, which was found to be repeated 11 times in a 1.35-kb sequence and 8 times in a 360-bp sequence and which Hunger et al. considered to be an origin of replication. Although we found this motif repeated six times in the sequence of pWM11, it was not present in the 185-bp fragment. Several hypotheses could explain the function of this insert. The 185-bp fragment contained a 30-bp region at one end which could form three different stem and loop structures, while another palindromic sequence was found at the opposite end. This fragment could encode an RNA transcript which might be required for initiation of replication, as has been shown for ColE1 replicons (27). In addition, effects of RNA secondary structure have been described for such replicons (25). Alternatively, the insert might contain a binding site(s) normally recognized by a factor supplied in *trans* from either the chromosome or the indigenous plasmids of *A. calcoaceticus* BD413. Finally, it is perhaps significant that the first 120 bp of the 300-bp open reading frame were located in the 185-bp region.

In this study we demonstrated the utility of the shuttle plasmids (i) by showing that these vectors are stably maintained and are unmodified even after a number of cycles of inter- and intraspecies transformations and (ii) by cloning the ADH regulon from *A. lwoffii* RAG-1 and showing that either *E. coli* or suitable mutants of *A. lwoffii* RAG-1 could be used as recipients in complementation and transformation experiments. More recently, these shuttle plasmids have been used in other studies involving gene expression and transformation in systems such as emulsan production and cell surface esterase activity in RAG-1 (1). Experiments are currently in progress to determine the host range of the shuttle plasmids with a view toward extending their use as cloning vehicles in other *Acinetobacter* strains.

ACKNOWLEDGMENTS

We thank R. Avigad for excellent technical assistance, R. Petter for supplying pRET-1 and help with the zymogram analysis, S. Rosenblatt for making available the electroporation apparatus and providing invaluable advice on its use, and G. Cohen, M. Mevarech, S. Mendelovitz, D. Schiffman, and I. Tzarfati for helpful suggestions and discussions.

W. Minas was supported by a MINERVA predoctoral fellowship. This project was supported in part by a grant from Petroferm U.S.A.

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