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Transformation of *Acinetobacter calco-aceticus* (*Bacterium anitratum*)

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A highly efficient transformation system has been demonstrated in a strain of *Acinetobacter calco-aceticus* (*Bacterium anitratrum*). During mixed growth of various stable, unencapsulated, mutant strains, deoxyribonucleic acid (DNA) is liberated and fully encapuslated transformants can be isolated. Purified DNA preparations have been used to transform suitable recipient mutant strains for ability to synthesize capsules, ability to dispense with a growth factor requirement, and resistance to streptomycin. When the wild-type strain is deprived of its capsule, either by mechanical stripping or by mutation, the unencapsulated cells tend to form large clumped masses. A nonclumping mutant of an unencapsulated strain has been isolated. When ability to synthesize capsules is transformed into this nonclumping strain, the resultant cells no longer form chains, unlike the wild-type encapsulated strain. It appears likely that the occurrence of transformation during growth of mixed cultures, with glucose or gluconate as the carbon source, may be the result of osmotic rupture resulting from the inability of unencapsulated strains to oxidize triose phosphates as fast as they are formed. The finding of transformation in *Acinetobacter* may provide an additional useful organism for the study of this mode of genetic transfer since this strain grows well in a simple mineral medium containing a single oxidizable source of carbon. Furthermore, no special supplementary factors seem to be required for transformation to take place.

Previous studies in this laboratory have been concerned with the biosynthesis of the polysaccharide capsule of a gram-negative coccus isolated from the soil (8, 12, 13, 14). Very recently, this organism has been classified as a strain of *Acinetobacter calco-aceticus* (*Bacterium anitratum*). Several unencapsulated mutants of this organism were isolated in an attempt to determine some of the metabolic intermediates of capsule synthesis. These mutants were grown together in pairs, on semisolid media, to detect possible cross-feeding of intermediates. Such cross-feeding might result in capsule formation by one of the two paired strains that was itself unable to synthesize an intermediate excreted by the second paired strain. Microscopic examination after growth of the various mixed mutant pairs did indeed reveal the presence of encapsulated cells. Compared with the number of unencapsulated cells, however, the number of encapsulated organisms was relatively small. Furthermore, the encapsulated organisms seen had large capsules characteristic of the wild-type strain. If cross-feeding were responsible for the appearance of encapsulated cells, it would be expected that approximately half of the cells would be encapsulated, assuming equal growth rates for the mixed mutants. Also, cross-feeding might have resulted in smaller capsules since metabolic intermediates could diffuse away into the semisolid medium as well as entering those cells capable of converting these intermediates to capsular polysaccharide.

Because each of the unencapsulated mutants used was shown to be stable, and not to revert spontaneously to the encapsulated form, it was assumed that genetic recombination must have occurred between the two unencapsulated mutants which were grown together. This hypothesis was verified by streaking the mixed culture and showing that it was possible to isolate colonies composed entirely of stable encapsulated cells. It was finally established that transformation was responsible for the recombination observed.

In this paper, we describe some of our initial observations regarding transformation in *A. calco-aceticus*. Making use of the property of genetic recombination via transformation in this organism, we have been able to isolate an encapsulated strain that differs considerably from the wild-type organism in that it is no longer

able to form chains. A preliminary report of this work has been presented (E. Juni and A. Janíková, Bacteriol. Proc., p. 60, 1968).

MATERIALS AND METHODS

Organism and growth conditions. The organism used in these studies (*A. calco-aceticus*, strain BD4) was isolated from the soil by the enrichment culture technique with 2,3-butanediol as the sole carbon and energy source. Many of the properties of strain BD4 have been described previously (12). Cells were grown at 30 C either on the surfaces of plates containing 0.5% glucose, one-half the concentration of the salts in the S-2 medium of Monod and Wollman (10), and 2% agar, or with shaking in the same medium without the agar. Capsules were observed in wet mounts in India ink (6). Photographs were taken under oil immersion with a Bausch and Lomb phase-contrast microscope.

Selection of mutants. Mutagenesis was performed either by treatment with ultraviolet light from a germicidal lamp (100 μw/cm^2 for 15 to 50 min) or with 10^{-4} M acridine orange for 1 hr. Surviving cells were plated on the glucose-mineral medium described above, and unencapsulated mutants were selected on the basis of colonial morphology. Unlike wild-type cells, which form large mucoid colonies, unencapsulated mutants form smaller colonies which have a relatively rough and dry appearance. A streptomycin-resistant mutant (BD43str^r), resistant to 200 μg of streptomycin per ml, and an uncharacterized auxotroph were obtained after ultraviolet irradiation of the unencapsulated mutant strain BD43.

A nonclumping mutant (strain BD45NC) was obtained after ultraviolet irradiation of a cell suspension of the unencapsulated mutant strain BD45. Surviving cells were grown in liquid glucose-mineral medium with shaking. After 24 hr, the growth flask was removed from the shaker and permitted to stand without agitation for about 1 hr. The great majority of the cells formed large clumps which settled rapidly to the bottom of the flask. A small volume of the slightly turbid liquid near the surface of the medium, which contained cells that did not clump readily, was inoculated into a fresh batch of liquid medium and the process was repeated a total of four times. Although there were still clumping cells present after the fourth transfer, some of the surface liquid from the last flask was spread for colony isolation on the surface of a glucose-mineral-agar plate. After suitable incubation, two distinct colony types were noted, the first being identical to the starting strain BD45. The second colony type was shown to be made up of nonclumping cells since cell paste from this colony suspended very readily and wet mounts showed all the cells to be separated from each other. By contrast, cells of BD45, as well as most of the other unencapsulated mutant strains, always showed large, clumped masses of cells even after extensive trituration prior to microscopic examination.

Preparation of deoxyribonucleic acid (DNA) and transformation procedure. DNA was prepared by the method of Marmur (9) from early stationary-phase cells (12 hr) grown in the liquid medium described above. Transformation was routinely demonstrated using the "plate method" (7). Unencapsulated mutants were smeared over an area of approximately 1 cm^2 of a glucose-mineral-agar plate and covered with one drop of a solution of transforming DNA (0.1 to 1.0 μg). After incubation at 30 C for 24 hr, wet mounts in India ink were examined for the appearance of encapsulated cells. When viewing cells that were poorly (or not at all) transformed, the wet mounts were observed by means of a total magnification of 210-fold. In this way, each microscopic field could include thousands of unencapsulated cells and still reveal the presence of even a single encapsulated cell, which was clearly visible at this low magnification.

An uncharacterized auxotroph was transformed to prototrophy by the use of DNA from an unencapsulated prototrophic mutant (strain BD41) by adding DNA to a suspension of the auxotroph and plating large numbers of cells on the glucose-mineral medium. The same number of cells not treated with DNA was plated as a control. Transformation was evident by the significantly greater number of prototrophic colonies for the DNA-treated sample compared with the control.

Transformation for the streptomycin resistance character was performed by mixing streptomycin-sensitive cells with DNA from a streptomycin-resistant mutant (strain BD43str^r) on the surface of a glucose-mineral-agar plate and incubating for 24 hr at 30 C. At this time, some of the cell paste was spread on the surface of a similar plate containing 200 μg of streptomycin per ml. In contrast to untreated cells, those cells incubated with DNA from strain BD43str^r gave rise to numerous colonies on the streptomycin-containing plate.

Capsule stripping. Capsules were removed from encapsulated cells by suspending the organism, grown on glucose-mineral-agar plates, in water and subjecting the suspension to three successive passes through a glass chromatogram sprayer, as described previously (8). When desired, stripped cells were removed from capsular polysaccharide and any remaining encapsulated cells were removed by centrifugation at 3,000 $\times$ g for 15 min followed by washing of the hard packed pellet once with 0.1 M potassium phosphate buffer (pH 7.0). To observe massive cell clumping after removal of capsules, the stripped cell suspension was allowed to stand at room temperature for 1 to 3 hr without further treatment.

Enzymes, chemicals, and chemical methods. Deoxyribonuclease and ribonuclease were obtained from Worthington Biochemical Corp., Freehold, N.J. Lipase and Pronase were obtained from Sigma Chemical Co., St. Louis, Mo., and Calbiochem, Los Angeles, Calif., respectively. All chemicals used were obtained from commercial sources. Cell-free capsular polysaccharide, obtained after capsule stripping, was hydrolyzed to its constituent sugars, which were identified by paper chromatography as described previously (12).

Agglutinating antiserum. Antiserum capable of agglutinating encapsulated cells of *A. calco-aceticus* was obtained from rabbits that had been subjected to a

series of three weekly intravenous injections of approximately 5×10^7 heat-killed cells (60 C for 30 min) of wild-type strain BD4. Addition of a small drop of undiluted antiserum to a suspension of encapsulated *Acinetobacter* cells resulted in a rapid agglutination clearly visible to the unaided eye. Microscopically, encapsulated cells treated with diluted antiserum showed a Quellung reaction. Cells of an unrelated encapsulated strain of *Acinetobacter* (*B. anitratum*, ATCC no. 15149) were not agglutinated by this antiserum.

RESULTS

Initial observation of genetic recombination. The wild-type strain of *A. calco-aceticus* (strain BD4) has a large polysaccharide capsule (12; Fig. 1A). Unencapsulated mutants generally form large, not easily dispersed clumps of cells during growth in either liquid or semisolid media (Fig. 1B). Most of the unencapsulated mutants used in this study clumped in the same manner and could not be distinguished from each other on the basis of microscopic examination. Various mutant pairs were mixed together on the surface of a glucose-mineral agar plate and incubated at 30 C for 36 hr. When wet mounts in India ink were examined microscopically, it was noted that many of the mixed pairs contained a relatively small number of fully encapsulated cells (Fig. 1C). Independent controls, consisting of the growth of each unencapsulated mutant by itself on the same medium, never revealed the presence of revertants to the encapsulated form. In fact, those mutants which were stable and did not revert were specifically chosen for these studies, although several revertible strains were isolated during the course of mutant selection. Upon streaking mixed unencapsulated cultures for colony isolation, it was possible to find mucoid colonies composed entirely of stable encapsulated cells. The results shown in Table 1 were obtained by growing 14 independently isolated unencapsulated mutants together in all possible pairs.

Demonstration of transformation. The above findings strongly implied that the appearance of stable encapsulated cells in the mixed cultures of unencapsulated mutants was the result of genetic recombination. The most likely phenomena that could explain these results were either transduction or transformation. It was not possible to induce lysis in the mutant cultures upon exposure to small doses of ultraviolet light. It appeared unlikely, therefore, that this organism harbored a temperate phage capable of transducing genetic characters.

If transformation were responsible for the apparent recombination observed, some of the cells in the mixed growth must have lysed with the liberation of DNA which could then be taken up by cells of the second mutant type with resultant transformation of the recipient strain. When deoxyribonuclease (50 to 100 μg) was added to the unencapsulated mutants immediately after mixing on the agar surface, no encapsulated cells were observed after the usual incubation period. The effect of deoxyribonuclease in preventing the appearance of encapsulated recombinants was demonstrated with nine different unencapsulated mutant pairs which had consistently shown encapsulated cells when grown together in the absence of added deoxyribonuclease.

That recombination in this system resulted from transformation was proven conclusively by isolating DNA from each unencapsulated mutant and showing that when this material was applied to other suitable unencapsulated mutant strains, many encapsulated cells could be seen after growth of the recipient strains. DNA from each strain was found to result in encapsulated recombinants only when mixed with those strains with which recombination had been observed upon mixed growth (Table 1). All transformations were reciprocal in that DNA from either member of a recombining pair (Table 1) was capable of transforming intact cells of the second organism. No transformations were observed when DNA preparations were incubated with deoxyribonuclease (1 mg/ml) for 3 min prior to mixing with mutant strains that are normally capable of being transformed.

Transformation of other markers. In addition to transformation involving capsule synthesis, it has been possible to transform a streptomycin resistance marker to sensitive, unencapsulated strains. The wild-type strain BD4 has also been transformed for the streptomycin resistance marker, thus demonstrating that the presence of capsule does not prevent transforming DNA from entering the encapsulated cell. An uncharacterized auxotrophic mutant, capable of growing on the glucose-mineral medium supplemented with vitamin-free casein hydrolysate but unable to grow on the glucose-mineral medium alone, was transformed to prototrophy by DNA from the strain from which the auxotroph was derived.

Transformation of a nonclumping mutant for capsule synthesis. The wild-type strain of *A. calco-aceticus* (strain BD4) as well as the unencapsulated mutants described above are not completely suitable for quantitative transformation studies; the wild-type strain forms chains and the unencapsulated mutants grow in large, clumped masses of cells. A nonclumping mutant

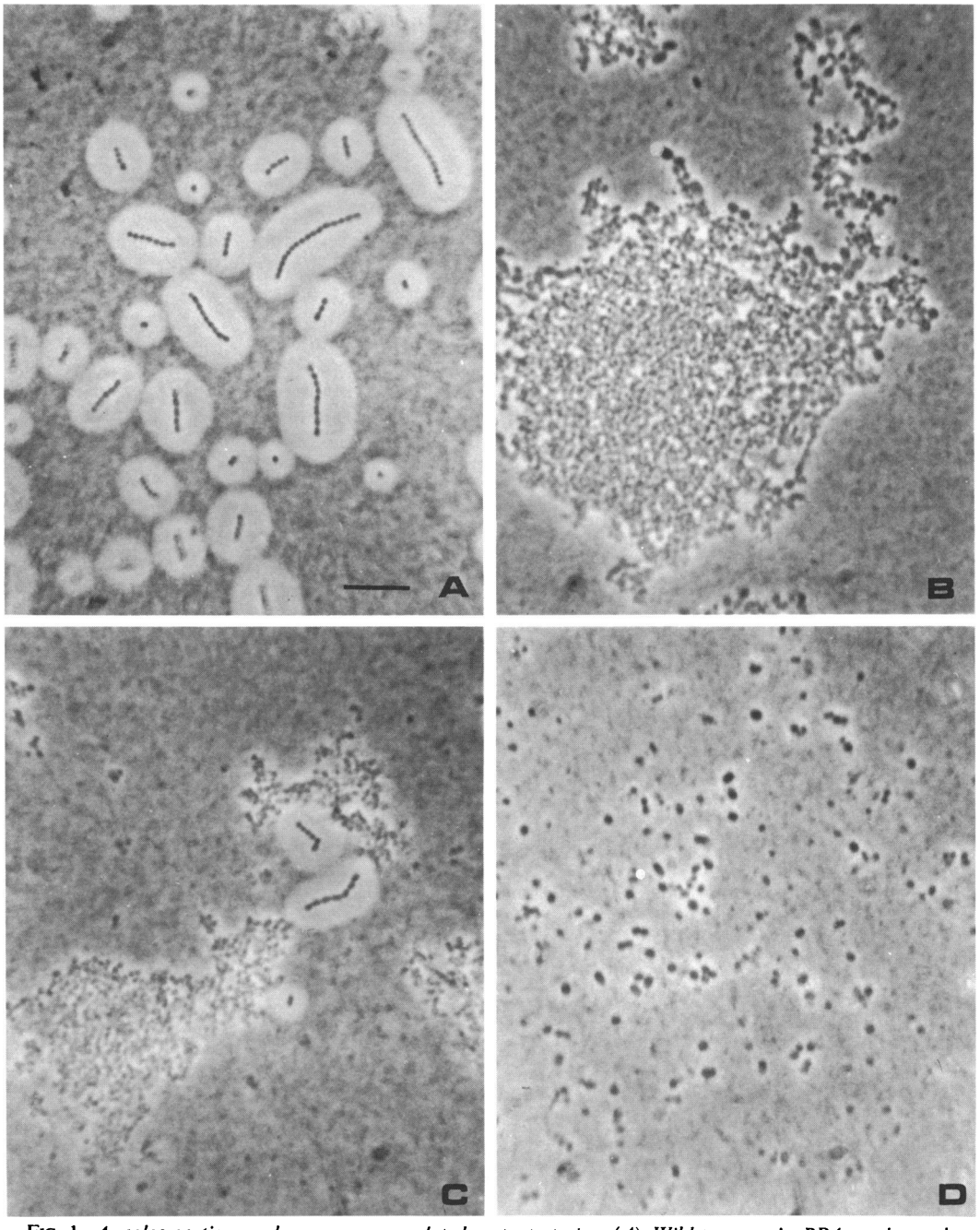

FIG. 1. *A. calco-aceticus and some unencapsulated mutant strains. (A) Wild-type strain BD4; scale marker, 10 μm. (B) Unencapsulated strain BD45. (C) Mixed growth of unencapsulated strains BD42 and BD45. (D) Non-clumping strain BD45NC.*

of the unencapsulated strain BD45 was obtained as described in Materials and Methods. When cell paste of the nonclumping mutant (strain BD45NC) was suspended in water and observed microscopically, the cells were found to be completely dispersed (Fig. 1D), unlike the parent strain (BD45) which showed large masses of clumped cells (Fig. 1B).

When wild-type strain BD4 is stripped of its capsule, the resulting unencapsulated cells tend

TABLE 1. *Formation of encapsulated recombinants after mixed growth of pairs of unencapsulated strains derived from wild-type strain BD4*[a]

Strain	BD41[b]	BD42[b]	BD43	BD44	BD45	BD46	BD47	BD48	BD410[b]	BD411	BD412	BD413	BD414	BD415[b]
BD415	+	+	+	+	±	+	+	+	−	+	+	+	+	−
BD414	+	+	+	±	±	±	+	+	+	+	+	±	−	
BD413	+	+	+	±	+	+	±	+	+	±	+	−		
BD412	±	+	+	+	+	+	+	+	+	−	−			
BD411	±	−	±	±	±	±	+	±	+	−				
BD410	±	+	+	+	+	+	+	+	−					
BD48	+	+	−	−	−	−	−	−						
BD47	+	+	−	−	−	−	−							
BD46	±	+	−	−	−	−								
BD45	+	+	−	−	−									
BD44	+	+	−	−										
BD43	+	+	−											
BD42	+	−												
BD41	−													

[a] Strains listed in the vertical heading were grown together with strains listed in the horizontal heading for 24 to 36 hr on the surface of a glucose-mineral-agar plate. The designation + indicates that encapsulated recombinants could be seen in almost every microscopic field examined. The designation ± is used for those cases where encapsulated cells were very rarely seen. When not even a single encapsulated cell could be seen after examining many fields, the symbol − is used. Each growth mixture was made and examined at least twice.

[b] These strains were obtained by the use of acridine orange mutagenesis; other strains were isolated after treatment with ultraviolet light.

to clump in large masses (8; Fig. 2A). It was considered, therefore, that it might be possible to transform the nonclumping strain BD45NC for capsule formation and thereby obtain a fully encapsulated organism which would not clump upon mechanical removal of capsules. Accordingly, strain BD45NC was treated separately with DNA from strains BD41 and BD42, both unencapsulated and clumping mutants. Encapsulated transformants were isolated in each case. When the encapsulated transformant (strain BD45NC1), transformed with DNA from strain BD41, was stripped of its capsules, the cells clumped in a manner entirely analogous to the clumping of stripped cells of wild-type strain BD4 (Fig. 2A). Before capsule stripping, strain BD45NC1 was microscopically indistinguishable from strain BD4 (Fig. 1A). By contrast, however, unencapsulated cells, obtained after mechanical stripping of the encapsulated transformant BD45NC2, which was derived by the use of DNA from strain BD42, failed to clump (Fig. 2B). Microscopic examination of strain BD45NC2, prior to capsule stripping, revealed a new and entirely different kind of encapsulated organism (Fig. 2C). A rather striking aspect of strain BD45NC2 (Fig. 2C) is the lack of chain formation characteristic of the wild-type strain BD4 (Fig. 1A). Examination of many microscopic fields failed to reveal any chains containing more than two cells. The sizes of the capsules of strain BD45NC2 were comparable to the sizes of the capsules of wild-type strain BD4 which surround one or two cells.

Upon forcible passage of an encapsulated cell suspension through a sprayer, as described in Materials and Methods, the stripped, unencapsulated cells of the wild-type strain BD4 tend to clump and form masses that not only are visible under the microscope (8; Fig. 2A) but also are large enough to be seen with the unaided eye (Fig. 2D). As anticipated, however, no clumping cell masses could be seen in the stripped cell suspension of strain BD45NC2 (Fig. 2D). Analysis of the capsular material derived from strain BD45NC2 revealed that it was composed entirely of rhamnose and glucose, the constituent sugars of wild-type strain BD4 capsular polysaccharide.

Cells of strain BD45NC2, as well as other encapsulated recombinant strains, were agglutinated readily by antiserum to wild-type strain BD4 cells.

Efficiency of transformation and periods of competence. Preliminary experiments have demonstrated that transformation in *Acinetobacter* is highly efficient. From 0.1 to 0.7% of auxotrophic cells in the early stationary phase of growth have been transformed to prototrophy by the use of 1 to 10 μg of wild-type donor DNA

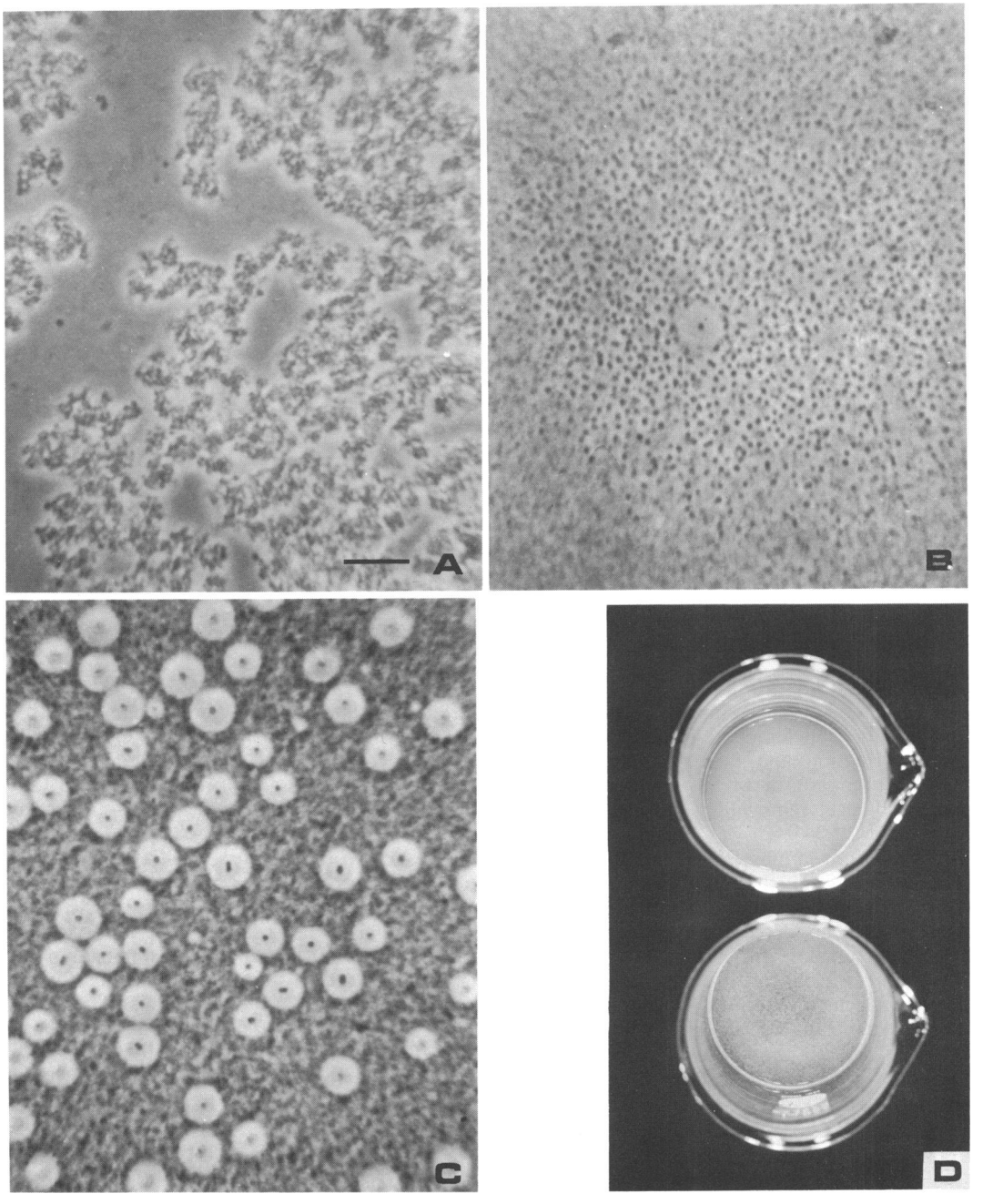

FIG. 2. *Capsule-stripping of wild-type strain BD4 and the transformed nonclumping strain BD45NC2. (A) Stripped cell suspension, strain BD4; scale marker, 10 μm. (B) Stripped cell suspension, strain BD45NC2. (C) Unstripped cell suspension, strain BD45NC2. (D) Uncentrifuged stripped cell suspensions in 250-ml beakers; lower beaker, strain BD4; upper beaker, strain BD45NC2.*

per ml. Transformation is less efficient during the logarithmic phase of growth and also falls off considerably when the stationary phase is reached. Quantitative studies of transformation in *Acinetobacter* are now in progress and will be described in detail in another publication.

DISCUSSION

The demonstration of transformation in strain BD4 and the unencapsulated mutants derived from this strain was made several months before this organism was definitively classified as a strain of *A. calco-aceticus* (*B. anitratum*). It was already known that this organism is oxidase-negative (12) and that it is, therefore, a member of the oxidase-negative *Moraxella* group (2). Although many of the characteristics of strain BD4 are typical of this group, strain BD4 is unusual in that it can grow with glucose or gluconate as the sole source of carbon and energy in an unsupplemented mineral medium. In a recent extensive study of the oxidase-negative moraxellas (2), only one of the 106 strains tested by these workers was able to use glucose or gluconate as the sole carbon source, although many of the strains were able to oxidize glucose and other sugars to their corresponding aldonic acids.

Transformation has been reported for various oxidase-positive moraxellas (3, 5). Although inter-species transformation has been observed for these organisms, DNA from various oxidase-negative moraxellas was not able to significantly transform the oxidase-positive strains (4, 5). Furthermore, the oxidase-negative species tested were not capable of being transformed by the use of homologous DNA (5).

The original finding of transformation in *Acinetobacter*, as demonstrated above, was made by growing various stable unencapsulated mutants together and observing the appearance of encapsulated recombinants. These results can be explained in terms of lysis of some of the cells during growth and the liberation of transforming DNA. Although recombination with suitable mutant pairs readily occurs when glucose or gluconate is the carbon source, virtually no recombination takes place between growing cells when 2,3-butanediol is the carbon source. These results may possibly be explained by the finding that growth on glucose or gluconate tends to produce cells that are larger, and probably swollen, when compared with cells grown on other carbon sources. Such swelling may, in fact, lead to disruption of some of the cells. One of the unencapsulated mutants used in this study (strain BD41) was shown to produce rather bizarre cells when grown on glucose or gluconate, but not when grown on 2,3-butanediol or nutrient agar. Many of these cells were as much as 10 to 50 times as large as cells grown on other carbon sources, and the largest of these appeared to be devoid of their cytoplasmic contents since they no longer appeared opaque when viewed with the phase-contrast microscope. An explanation of this effect of glucose is based upon the known carbohydrate metabolism of this organism. Glucose has been shown to be oxidized to gluconate, which is then degraded exclusively via the 2-keto-3-deoxy-6-phosphogluconate pathway with carbons 1, 2, and 3 of glucose, giving rise to pyruvate and carbons 4, 5, and 6 being converted to triose phosphate (13). In the wild-type encapsulated strain, most of the triose phosphate, formed from glucose or gluconate, is converted to capsular polysaccharide (8, 14). In the unencapsulated mutant strains, triose phosphate is metabolized to phosphoenolpyruvate, which is oxidized only after fixation of CO_2 to form oxalacetate. Since pyruvate kinase is lacking in this organism (13), fixation of CO_2 must precede further oxidation of phosphoenolpyruvate. It is possible that the rate of CO_2 fixation may not be great enough to remove phosphoenolpyruvate as rapidly as it is formed, and the accumulation of phosphorylated intermediates may result in osmotic rupture of some of the cells. In the case of strain BD41, there may also be an additional defect in the CO_2-fixing enzyme with a resultant extreme osmotic effect. The basis of this anomaly in strain BD41 is currently under investigation.

All of the unencapsulated strains used in these studies were completely stable and never reverted to the encapsulated form during a period of about 1 year after they were isolated. A transformation test was also made with two independently isolated, unencapsulated mutants, which had been carried in our stock culture collection for 6 years, by means of bimonthly transfers on nutrient-agar slants. When these two mutants were grown together, numerous encapsulated cells could be seen, and streaking of the mixed growth gave mucoid colonies consisting entirely of encapsulated cells. Examination of each of the mutant strains grown alone failed to reveal even a single encapsulated cell. These results emphasize the stability not only of the unencapsulated mutants but also of the property of transformability.

The ability of two unencapsulated mutants to transform each other for capsule synthesis is similar to the finding that two R-mutants of type I pneumococcus can inter-transform to give

a fully encapsulated type I organism (1). The fact that the various type I mutants used by these workers were either deficient in the same or in a different enzyme activity involved in capsular polysaccharide synthesis demonstrates that recombination within a cistron or between different cistrons can account for restoration of capsule-synthesizing capability.

It is characteristic of the wild-type strain BD4 that many of the cells form chains (12; Fig. 1A). When capsules are removed, the denuded cells tend to clump in large masses (8; Fig. 2A, 2D). It seems likely, therefore, that the tendency to form chains is a manifestation of clumping ability, the presence of capsules preventing random clumping and only permitting cells to remain joined in the region of cell separation after cell division. The nonclumping mutant strain BD-45NC (Fig. 1D) should, therefore, be unable to form chains. When strain BD45NC was transformed for ability to produce capsules, with DNA from strain BD42, the resulting transformant (strain BD45NC2) did indeed fail to form chains (Fig. 2C).

Since a clumping bacterium is not particularly suitable for quantitative transformation studies, strain BD45NC was isolated in the hope that individual cells would be separated from each other in liquid culture. Although this strain grown on semisolid media is dispersed readily in water and shows no clumps, it does, nevertheless, still have some clumping capacity when grown in liquid culture. Preliminary studies on the nature of the linkages involved in clumping have failed to reveal the basis of this phenomenon. Unlike the situation in *Corynebacterium xerosis* (11), clumping of unencapsulated mutant strains of BD4 is not affected by temperature, *p*H, or the action of a proteolytic enzyme (Pronase). Lipase, deoxyribonuclease, and ribonuclease also failed to disperse clumped masses of cells.

Examination of the various unencapsulated mutants isolated in this study has shown that strains BD413 and BD414 actually possess extremely small capsules. Strain BD413 can be grown in liquid culture without clumping, and studies with this strain are now in progress in an attempt to find the optimal conditions for competence as well as the maximal percentage of transformation possible with this organism. The simple nutritional requirements of *Acinetobacter*, as well as the lack of requirements for special supplementary factors for transformation, may make this organism particularly useful. It is also of interest that strain BD4 appears to grow equally well at 30 and 37 C.

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