APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1979, p. 316-323
0099-2240/79/02-0316/08$02.00/0000

Vol. 37, No. 2

Characterization of Plasmid Deoxyribonucleic Acid in *Streptococcus lactis* subsp. *diacetylactis*: Evidence for Plasmid-Linked Citrate Utilization†

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Received for publication 6 November 1978

The use of *Streptococcus diacetylactis* as a flavor producer in dairy fermentations is dependent upon its ability to produce diacetyl from citrate. Treatment of *S. diacetylactis* strains 18-16 and DRC1 with acridine orange resulted in the conversion of approximately 2% of the DRC1 population and 20% of the 18-16 population to citrate negative, which is indicative of the involvement of plasmid deoxyribonucleic acid (DNA). Growth in the presence of acridine orange also resulted in the appearance of 2% lactose-negative derivatives in *S. diacetylactis* 18-16 and 99% lactose-defective, proteinase-negative derivatives in *S. diacetylactis* DRC1. Cesium chloride-ethidium bromide equilibrium density gradients of cleared lysate material from each strain revealed the presence of covalently closed circular DNA. Samples of this covalently closed circular DNA were subjected to agarose gel electrophoresis to determine the plasmid composition of each strain. *S. diacetylactis* 18-16 was found to possess six plasmids, of approximately 41, 28, 6.4, 5.5, 3.4, and 3.0 megadaltons (Mdal). *S. diacetylactis* DRC1 contained six plasmids, of approximately 41, 31, 18, 5.5, 4.5, and 3.7 Mdal. Variants of *S. diacetylactis* 18-16 which failed to produce acetoin plus diacetyl from citrate (citrate negative) were missing a 5.5-Mdal plasmid. Lactose-negative mutants of the same strain were devoid of a 41-Mdal plasmid. Lactose-defective, proteinase-negative mutants of *S. diacetylactis* DRC1 were missing a 31-Mdal plasmid. The citrate-negative mutants of *S. diacetylactis* DRC1 isolated in this study did not possess a 5.5-Mdal plasmid. Thus, we have evidence that there is a correlation between the ability to utilize citrate and the presence of a 5.5-Mdal plasmid. A relationship was also noted between lactose fermentation and proteinase activity and plasmid DNA in *S. diacetylactis*.

The ability of *Streptococcus lactis* subsp. *diacetylactis* to produce diacetyl from citrate has led to its widespread use as a flavor producer in many dairy fermentations. It is this unique characteristic which distinguishes the organism from other lactic streptococci, although the significance of this distinction as it relates to classification of the species is questionable.

Homofermentative lactic acid streptococci which ferment citrate and produce volatile acids, CO_2, and other products were originally isolated and termed *S. diacetilactis* by Matuszewski et al. in 1936 (19). Soon afterwards, Krishnaswamy and Babel concluded that the organism would best be considered a variant of *S. lactis* and thus contributed the name *S. lactis* subsp. *aromaticus* (13). At about the same time, Swartling suggested that the organism's distinct ability to produce diacetyl entitled it to be named *S. diacetylactis* (25). The isolation of genetic variants which have lost their ability to ferment citrate and thus resemble *S. lactis* has strengthened the arguments of those who prefer the current classification of this organism as *S. lactis* subsp. *diacetylactis* (2, 5, 12, 19, 21, 25). However, Collins and Harvey reported that citrate-negative (Cit^-) mutants of certain strains of *S. diacetylactis* retain the enzyme citritase (citrate lyase, EC 4.1.3.6) and thus are still biochemically distinguishable from *S. lactis* (2). Mostert also reported the isolation of citrate permease-negative mutants of *S. diacetylactis* (21).

In a recent survey of *S. diacetylactis* and other lactic streptococcal isolates from vegetation, soil, water, and dairy products, Mostert concluded that *S. lactis* is probably a genetic variant of *S. diacetylactis* which has lost its ability to ferment citrate (21). Thus, the author suggested that a more likely classification would be *S. diacetylactis* subsp. *lactis*.

† Scientific Journal Series paper no. 10486, Minnesota Agricultural Experiment Station, St. Paul, MN 55108.

It is obvious that with the acquisition of knowledge concerning genetic variation among lactic streptococci, it will become increasingly difficult to assign these bacteria to strict classifications. Recent investigations in this and other laboratories have revealed that plasmid deoxyribonucleic acid (DNA) plays a significant role in the metabolism of the lactic streptococci (15, 23). Both the lactose and the proteinase enzyme systems have been shown to be plasmid linked in *S. lactis* (16, 17, 23). It has been reported by Kneteman (12), Collins and Harvey (2), and more recently by Mostert (21) that *S. diacetylactis* can spontaneously lose the ability to ferment citrate. In fact, with the extreme rate of conversion from Cit^+ to Cit^- and the apparent inability to revert, Kneteman was puzzled at how the Cit^+ organism could even be found in nature. In 1972, McKay et al. (17) showed that acriflavine treatment of *S. diacetylactis* 18-16 resulted in the appearance of lactose-negative (Lac^-) derivatives, implying the involvement of plasmid DNA in lactose utilization. We have found that plasmid DNA is present in *S. diacetylactis* strains 18-16 and DRC1 and have examined the relationship between plasmid DNA and the instability of citrate and lactose metabolism in these organisms.

MATERIALS AND METHODS

Organisms. *S. lactis* subsp. *diacetylactis* strains 18-16 and DRC1 were obtained from our stock culture collection. Cultures were maintained and transferred twice weekly in sterile (121°C, 12 min) 11% (wt/vol) reconstituted nonfat dry milk fortified with 0.15% sodium citrate. *Escherichia coli* K-12 strains J5, J53, and C600 were obtained from J. H. Crosa, Department of Microbiology, University of Washington, Seattle. Plasmids isolated from these strains were used as reference mobility markers in agarose gel electrophoresis (20, 24). Strains of *S. diacetylactis* and their derivatives used in this study are described in Table 1.

Isolation of mutants. Cit^- mutants were isolated by using acridine orange, which selectively inhibits the replication of plasmid DNA (9). Broth cultures in late log phase were diluted to 10^3 cells per ml in M17-glucose (26) containing acridine orange concentrations ranging from 0 to 25 μg/ml. The tube with the highest acridine orange concentration exhibiting growth after 24 to 48 h was diluted and plated on lactose indicator agar (17). The rate of conversion from Lac^+ to Lac^- was thus determined, and Lac^- derivatives were purified. One hundred representative colonies (50% Lac^+, 50% Lac^-) were then picked into 11% milk containing 1% glucose, 0.25% milk protein hydrolysate, and 0.15% citrate. After overnight incubation at 21°C, production of diacetyl plus acetoin was measured by the use of the King test (10); cultures which resulted in a negative reaction were considered Cit^-.

Citrate fermentation studies. Cells and extracts used in citrate fermentation studies were prepared as described by Collins and Harvey (2). For intact and toluene-treated cells, cultures were grown for 24 h at 21°C in citrate broth (7). Cells were washed twice with 0.05 M tris(hydroxymethyl)aminomethane buffer, pH 7.0, and resuspended to a cell density of 40 mg/ml. Toluene treatment involved the addition of 0.1 ml of a toluene–acetone mixture (1:9, vol/vol) to 5 ml of cells for 10 min at 0°C. This volume of toluene–acetone was found to have no effect on the colorimetric reaction used to measure citrate.

TABLE 1. *Strains of S. diacetylactis used*

Strain no.	Relevant phenotype	Derivation (reference)
18-16	Lac^+ Cit^+	Parent culture
GK1	Lac^- Cit^+	Acridine orange treatment (this study)
GK2	Lac^- Cit^+	Acridine orange treatment (this study)
GK4	Lac^- Cit^+	Acridine orange treatment (this study)
GK5	Lac^- Cit^-	Acridine orange treatment (this study)
GK82	Lac^+ Cit^-	Acridine orange treatment (this study)
DRC1	Lac^+ Cit^+ Prt^+	Parent culture
DRC1-X	Lac^+ Cit^- Prt^+	Obtained from E. B. Collins (2)
GK10	Lac^+ Cit^- Prt^+	Acridine orange treatment (this study)
GK13	Lac^d Cit^+ Prt^-	Acridine orange treatment (this study)

Cell-free extracts of *S. diacetylactis* strains DRC1 and 18-16 and their mutants were prepared from 1-liter citrate broth cultures grown for 24 h at 21°C. Cells were washed twice with cold 0.5 M tris(hydroxymethyl)aminomethane buffer and resuspended in 5.0 ml of the same buffer. Cells were fractured by using the Eaton press (6). Extracts were treated overnight at 0°C with deoxyribonuclease and ribonuclease and centrifuged at 15,000 × *g* for 30 min. The supernatants were then stored at 0°C. The citrate uptake experiments were performed as follows. For intact and toluene-treated cells, 0.2-ml aliquots were incubated at 32°C with 0.8 ml of 0.5 M phosphate buffer containing 0.25 mM sodium citrate and 1.25 mM $MgSO_4$. The pH of the buffer was 6.5 for toluene-treated cells, and it was 5.0 for intact cells. At periodic intervals, samples were removed and analyzed for citrate by the method of Marier and Boulet (18).

Citrate utilization studies with cell-free extracts were performed in a similar manner. A 1.0-ml volume of extract protein (20 mg/ml) was incubated at 32°C with a 9.0-ml volume of 0.5 M phosphate buffer, pH 7.0, containing 0.25 mM sodium citrate and 2.5 mM $MgSO_4$. Samples were removed periodically and analyzed for citrate. The protein concentration of each extract was measured by the method of Lowry et al. (14).

Plasmid analysis. The presence of plasmid DNA was confirmed in each strain by the use of cesium chloride–ethidium bromide density gradient centrifugation. This method and subsequent procedures used for isolation and examination of plasmid DNA by

agarose gel electrophoresis were described previously (11).

RESULTS

Curing experiments. Variants unable to utilize citrate were found by Collins and Harvey to occur in cultures of *S. diacetylactis* (2). These authors reported that the Cit^- population could increase on continued daily propagation, eventually reaching levels that could impair the aroma-producing ability of the culture. They also found that incubation at 40°C served as a useful selection procedure for Cit^- mutants for two of the cultures examined. The Cit^- variants described by Collins and Harvey (2) were cryptic, i.e., defective in transporting citrate across the cell barrier (citrate permease-negative), but still retained citritase. The recent report by Mostert (21) also confirms the spontaneous occurrence of Cit^- variants in cultures of *S. diacetylactis*. The spontaneous appearance of these Cit^- derivatives and the effect of 40°C provide indirect evidence that the conversion from Cit^+ to Cit^- could be due to loss of plasmid DNA (22).

Although preliminary attempts in our laboratory to isolate spontaneous Cit^- cells from several strains of *S. diacetylactis* were unsuccessful, we found that acridine orange concentrations of 15 to 20 μg/ml yielded a high conversion rate. After growth in broth containing this curing agent, approximately 20% of the strain 18-16 survivors were Lac^- and about 2% were Cit^-. The phenotypes observed were Lac^- Cit^+, Lac^+ Cit^-, and Lac^- Cit^-. Treatment of DRC1 with acridine orange resulted in the partial loss of lactose-fermenting ability in about 99% of the population. These mutants were termed lactose defective (Lac^d), because they produced acid slowly from lactose on the indicator agar. These mutants also required an exogenous nitrogen source to rapidly coagulate milk (<16 h at 21°C) in the presence of glucose, indicating that they were also proteinase negative (Prt^-). Of the remaining Lac^+ colonies on the indicator plate, up to 50% were Cit^-, i.e., Lac^+ Cit^-.

Presence of plasmid DNA in *S. diacetylactis* strains 18-16 and DRC1. Cords et al. (4) previously provided physical evidence for the existence of plasmid DNA in Lac^+ and Lac^- cells of *S. diacetylactis* 18-16. The results presented in this section confirm the presence of plasmids in 18-16 as well as in DRC1. Tritiated thymidine-labeled DNAs from the two strains were subjected to CsCl-ethidium bromide density gradient centrifugation. The resulting profiles (Fig. 1) demonstrated the distribution of radioactivity in the gradients. In both strains, a satellite peak characteristic of plasmid DNA was observed (fractions 10 to 16 for 18-16 and fractions 20 to 27 for DRC1). Samples of plasmid DNA collected from CsCl-ethidium bromide gradients as well as those isolated by ethanol precipitation of cleared lysates were then subjected to agarose gel electrophoresis to determine the total number of plasmids in each strain. The results (Fig. 2) indicated that both strains contained at least six individual plasmids. Based on relative mobility curves with the *E. coli* reference plasmids (20, 24), DRC1 contained plasmid molecules of about 41, 31, 18, 5.5, 4.5, and 3.7 megadaltons (Mdal), whereas 18-16 contained plasmid molecules of approximately 41, 28, 6.4, 5.5, 3.4, and 3.0 Mdal.

Plasmid profiles of the mutant derivatives. Since it was shown above that distinct plasmid molecules existed in DRC1 and 18-16, it was of interest to determine which, if any, of these molecules might be missing in the acridine orange-derived mutants. Plasmid profiles of two

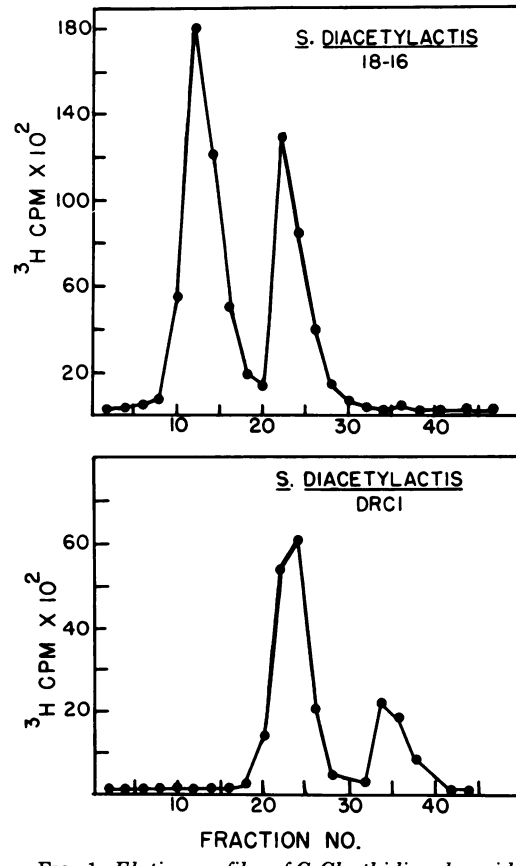

FIG. 1. *Elution profiles of CsCl-ethidium bromide gradients of cleared lysates from [^{3}H]thymidine-labeled S. diacetylactis strains 18-16 and DRC1.*

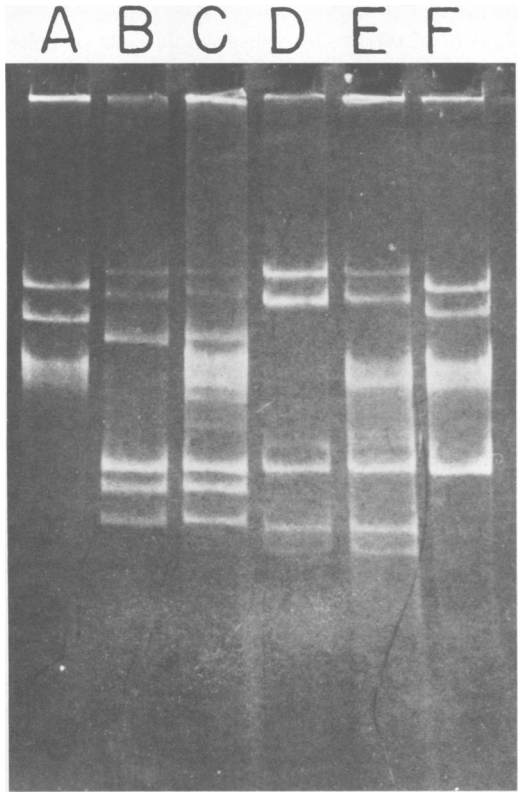

FIG. 2. *Agarose gel electrophoretic patterns of DNAs isolated from S. diacetylactis strains 18-16 and DRC1. Wells B and D contain plasmid DNA fractions collected from CsCl-ethidium bromide gradients. Wells C and E contain DNAs isolated from ethanol-precipitated cleared lysates. (A) E. coli mobility reference plasmids RP4 (34 Mdal) and Sa (23 Mdal). (B) S. diacetylactis 18-16 plasmids (top to bottom) pGK4101 (41 Mdal), pGK2801 (28 Mdal), pGK0641 (6.4 Mdal), pGK0551 (5.5 Mdal), pGK0341 (3.4 Mdal), and pGK0301 (3.0 Mdal). (C) S. diacetylactis 18-16 plasmids from ethanol-precipitated cleared lysates, demonstrating the same plasmids as (B) as well as a diffuse band seen in this and subsequent patterns which is composed of chromosomal fragments as described by Meyers et al. (20). (D) S. diacetylactis DRC1 plasmids pGK4102 (41 Mdal), pGK3101 (31 Mdal), pGK1801 (18 Mdal), pGK0552 (5.5 Mdal), pGK0451 (4.5 Mdal), and pGK0371 (3.7 Mdal). (E) S. diacetylactis DRC1 plasmids from ethanol-precipitated cleared lysates, demonstrating the same plasmids as (D) as well as chromosomal band. (F) E. coli reference mobility plasmids RP4 (34 Mdal), Sa (23 Mdal), and RSF1010 (5.5 Mdal). The molecular weights of S. diacetylactis plasmids were determined by migration relative to E. coli reference plasmids.*

Lac^- Cit^+ mutants of 18-16, designated GK1 and GK2, are shown in Fig. 3. Both mutants were missing the 41-Mdal plasmid. Figure 4 shows the agarose gel electrophoretic patterns of a Lac^+ Cit^- (GK82) derivative, another Lac^- Cit^+ (GK4) derivative, and a Lac^- Cit^- (GK5) derivative of 18-16; GK82 was missing only the 5.5-Mdal plasmid, GK4 lacked the 41-Mdal plasmid, and GK5 was missing both the 41- and the 5.5-Mdal plasmid species, as well as a 3.0-Mdal plasmid. These results suggest that lactose metabolism is mediated via the 41-Mdal plasmid and that citrate utilization is mediated via the 5.5-Mdal plasmid in *S. diacetylactis* 18-16. No correlation has yet been observed between loss of a phenotype and loss of the 3.0-Mdal plasmid.

A plasmid profile of GK13, a Lac^d Prt^- Cit^+ strain of *S. diacetylactis* DRC1, indicated that these mutants were missing the 31-Mdal plasmid (Fig. 5), whereas GK10, a Lac^+ Prt^+ Cit^- variant of DRC1, lacked the 5.5-Mdal plasmid. This

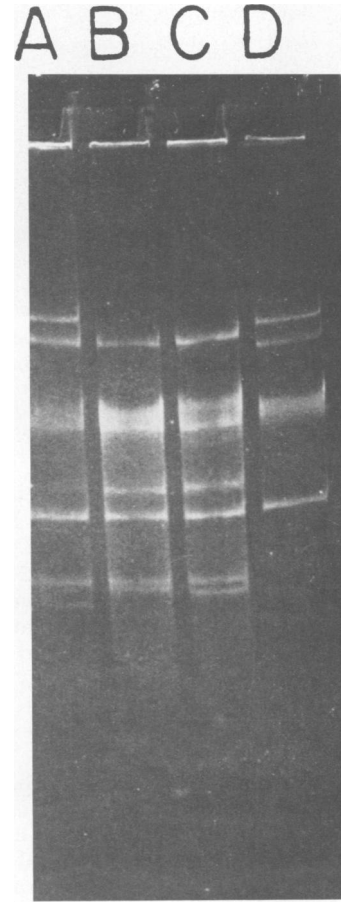

FIG. 3. *Agarose gel electrophoretic patterns of ethanol-precipitated DNAs from S. diacetylactis 18-16 (A) and Lac^- mutants GK1 and GK2 (B and C). Well D contains E. coli reference mobility plasmids RP4, Sa, and RSF1010.*

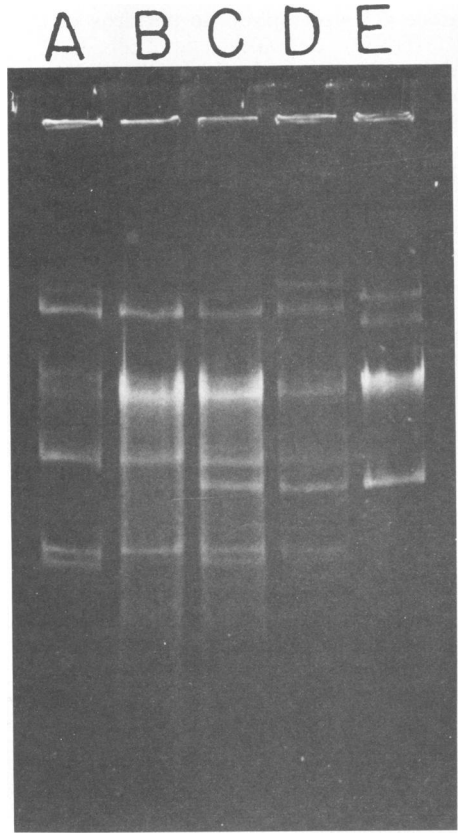

FIG. 4. *Agarose gel electrophoresis of ethanol-precipitated DNAs from cleared lysates of S. diacetylactis GK82 (Lac$^+$ Cit$^-$) (A), GK5 (Lac$^-$ Cit$^-$) (B), GK4 (Lac$^-$ Cit$^+$) (C), wild-type S. diacetylactis 18-16 (D), and E. coli plasmids RP4, Sa, and RSF1010 (E).*

result suggests that citrate utilization in DRC1 is also linked to a 5.5-Mdal plasmid. Figure 5 also shows the plasmid profile of DRC1-X, the Cit$^-$ mutant described by Collins and Harvey (2) which contains citritase but not the citrate permease. This mutant, which was isolated after repeated transfers in citrate broth, did not appear to be missing any of the plasmids present in the parent culture. A summary of the plasmid contents and designations of all strains examined is given in Table 2.

Citrate fermentation studies. The initial steps in citrate utilization by *S. diacetylactis* are the transport of citrate through the cell permeability barrier by a permease system (8) and the splitting of citrate into acetate and oxaloacetic acid by the enzyme citritase (7). Since DRC1-X was shown by Collins and Harvey (2) to be missing the citrate permease but not citritase, it was of interest to determine the nature of the Cit$^-$ mutants isolated in this study. Two representative Cit$^-$ cultures were first tested for utilization of citrate by using whole cells or cells treated with toluene to destroy the permeability barrier. Whole and toluene-treated cells of DRC1 utilized all of the citrate in the reaction mixture within the 60-min incubation period. This indicated the presence of both the citrate permease and citritase. On the other hand, whole cells of the Cit$^-$ variants GK82 and GK10, as well as DRC1-X, were unable to utilize any of the citrate, providing evidence that our mutants were also missing the citrate permease. When using toluene-treated cells, DRC1-X was observed to utilize all of the citrate in the reaction mixture within 60 min, whereas the Cit$^-$ mutants isolated herein appeared to utilize some, but not all, of the citrate in the same time period. The Cit$^-$ mutants isolated in this study, therefore, appeared to differ from DRC1-X. GK10 and GK82, which had lost the 5.5-Mdal plasmid,

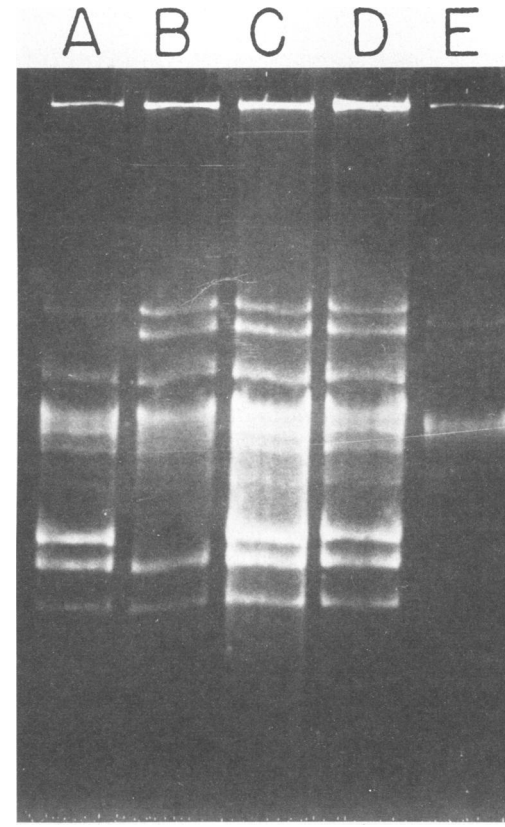

FIG. 5. *Agarose gel electrophoresis of ethanol-precipitated DNA from S. diacetylactis DRC1 and mutants. (A) S. diacetylactis GK13 (Lac$^-$ Prt$^-$ Cit$^+$); (B) GK10 (Lac$^+$ Prt$^+$ Cit$^-$), (C) DRC1-X (Lac$^+$ Prt$^+$ Cit$^-$), (D) DRC1, (E) E. coli plasmids RP4, Sa, and RSF1010.*

were not only missing the citrate permease but also appeared defective in citritase. To further clarify this difference, the utilization of citrate over time by cell-free extracts was measured with *S. diacetylactis* strains DRC1 and 18-16 and the Cit$^-$ mutants GK82, GK10, and DRC1-X. The results are presented in Fig. 6. The parent culture DRC1 utilized about 130 μg of citrate per mg of protein in 60 min. DRC1-X extracts utilized about 75 μg of citrate per mg of protein during the same time period, indicating that although it contained citritase, the activity was less than that observed in the parent culture. In contrast, extracts from GK10, which had lost the 5.5-Mdal plasmid, were considerably less active in utilization of citrate; only 25 μg of citrate per mg of protein was utilized in 60 min. Extracts from *S. diacetylactis* 18-16 utilized 264 μg of citrate per mg of protein in 60 min, whereas the deficient mutant, GK82, was able to utilize 180 μg of citrate per mg of protein in 60 min.

DISCUSSION

The metabolic properties of *S. diacetylactis* appear to be governed in part by DNA associated with extrachromosomal elements. At least six distinct plasmid species have been observed in both *S. diacetylactis* 18-16 and *S. diacetylactis* DRC1. Results presented in this communication provide evidence that lactose, proteinase, and citrate activity may all be associated with specific plasmids.

Lactose-fermenting ability and proteinase activity are thought to be plasmid mediated in *S. lactis* (16, 17, 23). In both strains of *S. diacetylactis* examined in this study, the ability to ferment lactose was highly unstable and was readily cured when cells were exposed to acridine orange. Proteinase activity was also readily cured in *S. diacetylactis* DRC1; the wild-type 18-16 used in this study appears to be Prt$^-$. Agarose gel electrophoretic patterns of plasmid DNA of *S. diacetylactis* 18-16 Lac$^-$ strains indicated a positive correlation between appearance of the Lac$^-$ phenotype and loss of the 41-

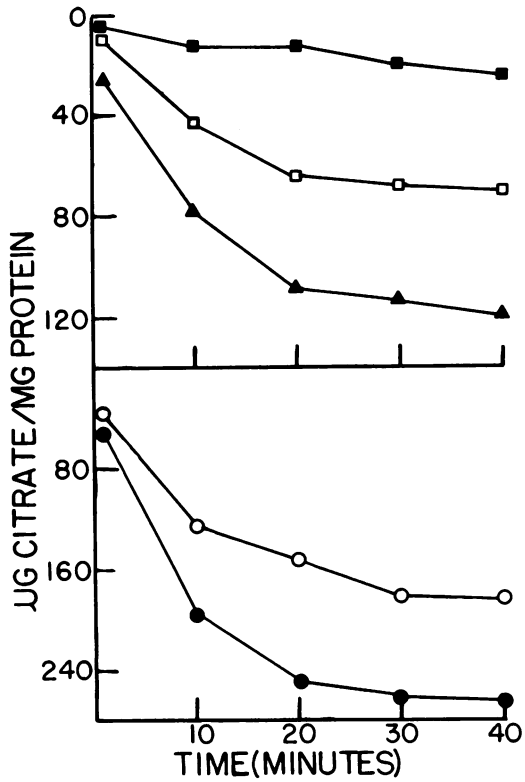

FIG. 6. *Citrate utilization by cell-free extracts of S. diacetylactis strains 18-16 and DRC1 and Cit$^-$ variants. Reaction mixtures contained sodium citrate, 0.25 × 10^{-3} M; MgSO$_4$, 2.5 × 10^{-3} M; pH 7.0 phosphate buffer, 0.5 M; 1.0 ml of extracts (20 mg of protein per ml); total volume, 10 ml. Symbols: ■, S. diacetylatis GK10; □, DRC1-X; ▲, DRC1; ○, GK82; ●, 18-16.*

TABLE 2. *Plasmid content of S. diacetylactis strains studied*

Strain no.	Relevant phenotype	Plasmid content of host strain[a]					
18-16	Lac$^+$ Cit$^+$	pGK4101	pGK2801	pGK0641	pGK0551	pGK0341	pGK0301
GK1	Lac$^-$ Cit$^+$		pGK2801	pGK0641	pGK0551	pGK0341	pGK0301
GK2	Lac$^-$ Cit$^+$		pGK2801	pGK0641	pGK0551	pGK0341	pGK0301
GK4	Lac$^-$ Cit$^+$		pGK2801	pGK0641	pGK0551	pGK0341	pGK0301
GK5	Lac$^-$ Cit$^-$		pGK2801	pGK0641		pGK0341	
GK82	Lac$^+$ Cit$^-$	pGK4101	pGK2801	pGK0641		pGK0341	pGK0301
DRC1	Lac$^+$ Cit$^+$	pGK4102	pGK3101	pGK1801	pGK0552	pGK0451	pGK0371
DRC1-X	Lac$^+$ Cit$^-$ Prt$^+$	pGK4102	pGK3101	pGK1801	pGK0552	pGK0451	pGK0371
GK10	Lac$^+$ Cit$^-$ Prt$^+$	pGK4102	pGK3101	pGK1801		pGK0451	pGK0371
GK13	Lac^d Cit$^+$ Prt$^-$	pGK4102		pGK1801	pGK0552	pGK0451	pGK0371

[a] Plasmid compositions were determined by agarose gel electrophoresis. Plasmids were designated as follows: the three digits following pGK represent plasmid molecular weight × 10^5; the fourth digit distinguishes plasmids from different parent strains having the same molecular weight.

Mdal plasmid. In *S. diacetylactis* DRC1, loss of proteinase activity and defective lactose-fermenting ability occurred concomitantly with the disappearance of a 31-Mdal plasmid. These data suggest that lactose-fermenting ability is associated with the 41- and 31-Mdal plasmids in *S. diacetylactis* strains 18-16 and DRC1, respectively. Lac^d mutants of *S. diacetylactis* DRC1 were also Prt^-. Thus, proteinase activity may also be linked to the 31-Mdal plasmid in DRC1.

Of further interest regarding *S. diacetylactis* DRC1 is the fact that Lac^d strains of the organism were able to utilize lactose slowly, and they strongly resembled the pseudorevertants reported by Cords and McKay (3). However, these Lac^d variants did not appear after prolonged incubation; rather, they were the immediate result of curing. This suggests that the organisms may have an alternate mechanism for lactose utilization, which could be carried on another plasmid species or on the chromosome. This alternate system could be immediately activated upon loss of a lactose plasmid or be active even in the presence of a lactose plasmid but not observed due to dominance of the normal *lac* genes.

Evidence that citrate-fermenting ability is plasmid linked comes from several observations: (i) citrate metabolism is unstable in certain strains of *S. diacetylactis*, (ii) the ability to utilize citrate is cured by the use of acridine orange, (iii) a 5.5-Mdal plasmid has been found to be missing in the Cit^- mutants, and (iv) citrate utilization by both intact cells and cell-free extracts is deficient in the Cit^- mutants which are missing the 5.5-Mdal plasmid.

Although several enzymes essential to citrate metabolism may be linked to the 5.5-Mdal plasmid, the evidence provided in this paper indicates that the loss of this plasmid results in loss of citrate permease activity.

When Collins and Harvey reported the instability of citrate metabolism in *S. diacetylactis*, they observed that cells which had lost the ability to transport citrate still retained citritase activity (2). This activity was lower than that of the parent, due to the fact that citritase production is repressed when cells are grown in the absence of citrate or when citrate is not transported into the cell (as in the case of permease mutants) (7). We have observed a similar response in the acridine orange-induced Cit^- mutants GK10 and GK82, both of which have lost the 5.5-Mdal plasmid. Although our mutants appeared to have less citritase activity than did DRC1-X, some activity was retained, indicating a probable repression of the enzyme rather than its loss. Thus, in both *S. diacetylactis* 18-16 and *S. diacetylactis* DRC1, the permease activity appears to be the only activity which is consistently lost with the loss of the 5.5-Mdal plasmid.

The fact that the mutant DRC1-X is Cit^- while retaining the 5.5-Mdal plasmid can probably best be attributed to a point mutation on the citrate plasmid. Similar findings have been reported by Anderson and McKay with regard to lactose metabolism (1). Although lactose-fermenting ability is associated with a 36-Mdal plasmid in *S. cremoris* B_1, spontaneous Lac^- derivatives were isolated which maintained this plasmid. The authors attributed this finding to a point mutation in enzyme II on the lactose plasmid. This spontaneous Lac^- derivative containing the lactose plasmid was able to revert full lactose-fermenting ability, whereas Lac^- mutants which had lost the 36-Mdal plasmid were able to revert only to a partial lactose-fermenting phenotype. Similarly, if strain DRC1-X contains a point mutation on the citrate plasmid, this strain should have the ability to fully revert. In contrast, strains GK10 and GK82, which have lost the 5.5-Mdal plasmid, should be unable to revert to the Cit^+ phenotype. This has not been examined due to the lack of a suitable selection medium for screening Cit^+ clones on a lawn of Cit^- cells. However, Kneteman reported that spontaneous Cit^- mutants did not revert to Cit^+ in milk or on a solid medium (12).

It is of interest that DRC1-X was isolated by repeated transfer in broth, a condition which normally leads to curing of specific plasmid DNA. However, the mutants appeared in citrate broth, which would tend to select for maintenance of the citrate plasmid rather than its loss. It would seem likely that DRC1-X is not representative of Cit^- mutants which arise in cultures of *S. diacetylactis*, and examination of other spontaneous Cit^- strains may reveal mutants which have lost the 5.5-Mdal plasmid. In any event, the results presented here strongly imply the involvement of the 5.5-Mdal plasmid with the ability of *S. diacetylactis* strains 18-16 and DRC1 to utilize citrate. These data also provide evidence that cells which have lost the 5.5-Mdal plasmid and subsequently become Cit^- still possess citritase activity and thus retain their distinction from *S. lactis*.

Current research in the development of genetic transfer systems should lead to further clarification of the role of plasmid DNA in *S. diacetylactis* strains and its relationship to citrate and lactose fermentation, as well as proteinase activity.

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