The Elimination of Urease Activity in *Streptococcus faecium* as Evidence for Plasmid-coded Urease

By A. R. COOK

The Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB

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SUMMARY

A strain of *Streptococcus faecium* from the sheep rumen showed spontaneous loss of urease activity when subcultured at the normal rumen temperature of 38 °C, although in mixed cultures *in vivo* or *in vitro* loss of urease was not apparent. The rate of loss of urease in pure cultures was increased at incubation temperatures above 38 °C, but loss was never complete. However, at temperatures below 38 °C loss was greater, and at 22 or 18 °C the urease was completely eliminated. Incubation with sodium dodecyl sulphate (0.002 %) or ethidium bromide (2.5×10^{-5} M) caused complete loss of urease activity. The urease activity was also eliminated when the streptococcus was grown aerobically, and this loss of activity was irreversible.

It is suggested that the urease activity is controlled by a plasmid gene and that aeration, low growth temperature and chemical agents 'cure' the streptococcus of the plasmid. Attempts to demonstrate the presence of covalently closed circular extrachromosomal DNA by caesium chloride-ethidium bromide equilibrium density-gradient centrifugation were unsuccessful.

INTRODUCTION

During the isolation of a ureolytic strain of *Streptococcus faecium* from the rumen of a sheep it was observed that the urease activity was an unstable property. Also, this nutritionally-fastidious streptococcus was unable to utilize ammonia as a source of nitrogen, and growth of either a urease-free strain or a ureolytic strain was not affected by addition of urea to a complex medium (Cook, 1976). Thus the presence of the urease was not essential for the survival of the bacterium. The results suggested that the ureolytic activity of the streptococcus might be coded on a plasmid (Cook, 1976).

The spontaneous loss of a plasmid genetic trait may be increased by growth under certain physical conditions and by growth in the presence of 'curing agents' (Novick, 1969; Clowes, 1972). The collective results of such experiments have been used to provide evidence that urease in *S. faecium* is coded on a plasmid. Plasmids exist as autonomously replicating DNA molecules physically separate from the host chromosome and in a covalently closed circular (CCC) form which has been isolated from several bacteria with extrachromosomal properties (Bazaraal & Helinski, 1968; Christiansen *et al.* 1973). An attempt was made to isolate a plasmid DNA species from the *S. faecium*.

METHODS

Bacteria. The ureolytic strain of *Streptococcus faecium* was isolated from the rumen of a sheep (Cook, 1976). *Proteus mirabilis* NCIB60 was obtained from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen. The ureolytic strain of *Peptostreptococcus provostii* was isolated from the rumen of a calf on a molasses-urea diet (Elias, 1971). Cultures were incubated at 38 °C unless otherwise stated.

Chemicals. Brain-heart infusion and urea agar base were from Oxoid. Bactocasitone, Bactopeptone, Bactotryptone, Bacto yeast extract and agar were from Difco. BRIJ-58 was from Serva and supplied by Micro-Bio Laboratories Ltd, London. All other chemicals were from BDH. Glass-distilled water was used throughout. Carbon dioxide (British Oxygen Company, London) was passed over heated copper to give an oxygen-free supply.

Media. The SBU medium for *S. faecium* contained, per 100 ml glass-distilled water: 0.1 ml resazurin (0.1 %), 0.5 g Bactocasitone, 0.5 g yeast extract, 0.2 g mannitol, 0.05 g anhydrous sodium acetate, 0.075 g sodium thioglycollate, 0.5 g KH_2PO_4 , 0.5 g Na_2HPO_4 , 0.2 g urea. The medium, without urea, was adjusted to pH 7.4, sterilized by autoclaving at 121 °C for 15 min, and a Seitz-filtered urea solution (1 ml of 20 %, w/v) was added under anaerobic conditions (oxygen-free CO_2). When necessary the medium was dispensed under CO_2 . The final pH was 6.8. Initially the medium was inoculated with a 2 % (v/v) inoculum and incubated for 24 h under CO_2 . In later experiments the inoculum size was reduced to 0.3 %. Other media used were SBY, which was similar to SBU except that urea was omitted, and SU and SY which were similar to SBU and SBY respectively except that Bactocasitone was omitted. The final pH of media SY and SBY was 6.6. Cultures were checked for purity by streaking a loopful on a brain-heart infusion agar plate which was incubated aerobically for 24 h.

The medium (PMU) used for *P. mirabilis* contained, per 100 ml: 0.75 g Na_2HPO_4 , 0.75 g KH_2PO_4 , 0.01 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g Bactopeptone, 1 ml nicotinic acid (0.1 %). The medium was adjusted to pH 6.5 and, after sterilization by autoclaving at 121 °C for 15 min, 1 ml of a Seitz-filtered solution containing urea and glucose was added to give final concentrations of 0.015 % glucose and 0.06 % urea. The medium was dispensed as 5 ml lots into 50 ml conical flasks, inoculated with a 0.6 % inoculum and incubated for 24 h on an orbital shaker (G24 environmental incubator shaker, New Brunswick Scientific Co., New Brunswick, New Jersey, U.S.A.). Culture purity was checked by microscopic examination.

Peptostreptococcus provostii was grown in medium AE which contained, per 100 ml: 0.1 ml resazurin (0.1 %), 7.5 ml mineral solution A (6 g $\text{K}_2\text{HPO}_4/\text{l}$), 7.5 ml mineral solution B (6 g KH_2PO_4 , 12 g NaCl , 1.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.2 g CaCl_2/l), 0.5 g yeast extract, 0.5 g Bactotryptone, 1 ml $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.34 %), 1 ml $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.15 %). After sterilization by autoclaving at 121 °C for 15 min, 10 ml Seitz-filtered solution R (0.2 g sucrose, 0.4 g NaHCO_3 , 0.05 g cysteine-HCl) was added and the complete medium dispensed in 10 ml portions under oxygen-free CO_2 . The medium was inoculated with a 0.3 % inoculum and incubated for 24 h under anaerobic conditions. Culture purity was checked by microscopic examination.

Colony-forming unit (c.f.u.) determinations. For *Streptococcus faecium* and *Proteus mirabilis*, these were carried out by making serial tenfold dilutions in brain-heart infusion broth and mixing 0.5 ml samples from the dilution in 10 ml brain-heart infusion agar in a 90 mm diameter Petri dish (Sterilin Ltd., Teddington, Middlesex) followed by a 48 h aerobic incubation. With *Peptostreptococcus provostii*, serial tenfold dilutions were carried out using a dilution fluid similar to medium AE except that $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and sucrose were omitted. A 0.5 ml sample of an appropriate dilution was added to 4.5 ml of medium AE with 2 % (w/v) agar in a test tube and a roll tube prepared which was incubated for 72 h. For any dilution the sampling was done in triplicate and only those plates or roll tubes with c.f.u. counts between 30 and 300 were considered significant.

Growth. Bacterial density was determined at 650 nm as previously described (Cook, 1976).

Urease activity. The units of urease activity (i.u./ml) were previously defined (Cook, 1976). A sample (1 ml) of culture was resuspended in either 2 or 4 ml of assay buffer (0.02 M-

sodium phosphate buffer pH 7.0, containing 0.001 M-EDTA) and 0.2 or 0.1 ml of cell suspension was assayed as previously described (Cook, 1976). A loopful of an isolate of *Peptostreptococcus provostii* on AE slopes was resuspended in 0.2 ml of assay buffer and assayed by the rapid method (Cook, 1976).

Inoculum size and culture urease activity. In one series, SY medium (10 ml) was inoculated with a 0.3 % inoculum from a 24 h culture grown in medium SU, and after incubation for 24 h the culture was the source of inoculum (0.3 %) for SY (10 ml) and SU (10 ml) media, which were then incubated for 24 h. The subculturing of the previous culture grown in SY medium into SY and SU media was repeated for up to 21 consecutive daily transfers. In another series, SBY and SBU media were used. The control in both series was an inoculum size of 2 % (v/v). After 24 h incubation the bacterial density was measured at E_{650} and the urease activity determined.

Temperature treatment. SU and SBU media were inoculated from a 24 h culture with a 0.3 % inoculum, and incubated for 24 h at temperatures between 18 and 48 °C, after which the urease activity and bacterial density were measured. At each temperature there were four successive subcultures, and when a culture was not ureolytic or when the urease activity was very low, then this culture was subcultured into the same medium and incubated at 38 °C. If the subculture grown at 38 °C was not ureolytic then up to 21 consecutive transfers were carried out, each incubated at 38 °C for 24 h. The control with either medium was incubated at 38 °C.

Sodium dodecyl sulphate (SDS) treatment. Medium SBU (10 ml), with concentrations of SDS from 0.0005 to 0.005 %, was inoculated with a diluted stationary-phase culture grown in SBU medium, to give a final viable count of 10^4 c.f.u./ml. After 24 h incubation the bacterial density, viable count and urease activity of each culture in which growth had occurred were determined, and for each of these cultures four consecutive daily subcultures (0.3 %) were made into SBU medium alone and SBU medium plus SDS at the same concentration. When a subculture in SBU medium alone was not ureolytic, 21 daily transfers on SBU medium were made to see if ureolytic activity was regained.

*Ethidium bromide (EB) treatment of *S. faecium*.* A small inoculum (to give 10^4 c.f.u./ml) from a diluted, overnight, stationary-phase culture grown in SBU medium was incubated in SBU medium (10 ml) containing EB at concentrations from 9×10^{-6} to 4×10^{-5} M. After overnight incubation the bacterial density, the viable count of those cultures with very low bacterial density and the urease activity were determined, and subcultures (0.3 % inoculum) made into SBU medium with or without the same concentration of EB. Subculturing from the EB-containing medium into media with and without EB was carried out on four consecutive days. If a culture without EB showed no urease activity the ability to regain activity was tested by 21 daily transfers, as with SDS treatment.

*Ethidium bromide treatment of *P. mirabilis*.* An inoculum from a diluted overnight culture in PMU medium (to give 4×10^4 c.f.u./ml) was added to flasks of PMU medium containing EB at concentrations from 5×10^{-5} to 5×10^{-4} M. The culture containing the highest concentration of EB in which visible growth had occurred in 24 h (culture A) was subcultured (0.3 % inoculum) into medium containing EB at the same concentration. Similarly, the cultures from the next higher and the next lower concentrations of EB were subcultured into media containing the same concentration of EB. After 24 h the three cultures were subcultured once more. Bacterial density and urease activity was determined in all cultures. Culture A and the first subculture from this were diluted in brain-heart infusion broth and 0.5 ml samples then dispensed in 10 ml brain-heart infusion agar containing 0.2 % urea, in a Petri dish, and incubated 48 h. One hundred isolated colonies were inoculated on to urea-

agar slopes and incubated 72 h. As a control the same procedure was used on a culture which had been grown without EB.

Ethidium bromide treatment of Peptostreptococcus provostii. Tubes of AE medium containing EB at concentrations from 2×10^{-6} to 4.5×10^{-6} M were inoculated (to give 1×10^4 c.f.u./ml) from a diluted culture grown in AE medium. After incubation for 24 h, the bacterial density and urease activity were determined to find the culture which had the highest concentration of EB in which growth occurred (culture B); a subculturing procedure, similar to that for *P. mirabilis*, was then followed for a total of four transfers, for this culture and those containing the next higher and lower concentrations of EB. Culture B was diluted and incubated in roll tubes for 72 h, after which one hundred colonies were isolated on to AE slopes, incubated for 48 h, and assayed for urease activity using the rapid method.

Aerobic growth of S. faecium. SBU medium (105 ml) was modified by omitting sodium thioglycollate and adding one drop of propane-1,2-diol as antifoaming agent. The medium was aerated overnight with a 1600 ml/min air flow humidified by passage through water. The air supply passing through the water and medium was fragmented by use of gas distribution tubes with domed sintered glass bulbs of porosity 0 (Sinta Glass, Gallenkamp, London). For 30 min before inoculation and during growth, the aeration rate was increased to 3000 ml/min and a 5 ml sample was removed as a blank for the measurement of bacterial density. The medium was then inoculated with a 0.3 % inoculum from an overnight culture grown in SBU. Samples (6 ml) were removed at 30 min intervals and after measuring the growth density the urease activity was determined by centrifuging a 5 ml sample, resuspending the sediment in 1 ml of assay buffer and assaying 0.4 ml for 30 min at 38 °C. At the sampling times, 2×10 ml lots of SBU medium, tubed under CO_2 , were inoculated (0.3 %) and incubated for either 24 or 48 h. The bacterial density of these cultures was measured and the urease activity determined by resuspending 1 ml culture, after centrifugation, in 1 ml assay buffer and assaying 0.2 ml for 5 min at 38 °C. In addition, the 24 h culture was subcultured into SBU medium and incubated anaerobically for 24 h, after which the growth density and urease activity were determined. Those anaerobic cultures which were not ureolytic were subcultured for up to 21 consecutive days.

Lysis and caesium chloride-ethidium bromide differential centrifugation. The method used was that of Courvalin, Carlier & Chabbert (1972). A 0.3 % inoculum of *S. faecium* was incubated in 100 ml SBU medium for 20 h. The cells were harvested by centrifugation at 0 °C and, after two cold washings with 0.85 % NaCl containing 0.005 M-EDTA at pH 7.0, the sediment was resuspended in 10 ml solution NE (2.5 M-NaCl and 0.05 M-EDTA at pH 7.0). To this suspension was added 1 ml lysozyme (400 µg/ml NE), and after incubation for 60 min at 37 °C the cells were lysed by adding 16.5 ml of solution BDE (1 % BRIJ-58, 0.04 % deoxycholate, 0.3 M-EDTA, pH 8.0) and gently swirling for 5 min at 20 °C. A sample (2 ml) of the supernatant obtained by centrifugation of the lysate at 50000 g for 15 min at 0 °C was mixed with 3 ml of 64 % (w/w) CsCl containing 550 µg ethidium bromide/ml and centrifuged in an L2-50 centrifuge (Beckman Instruments, Glenrothes, Fife) at 144000 g (r_{av} 7.3 cm) for 36 h at 15 °C using an SW 50L rotor. The tubes were examined in an ultra-violet light source (model LH 530; Gallenkamp, London).

This procedure was repeated using a culture inoculated (0.3 %) from a 20 h culture which had lost its urease activity following treatment with ethidium bromide at 3×10^{-5} M.

RESULTS

Effect of inoculum size on culture urease activity of S. faecium

Although urease activity in subcultures could always be retained by using a 2 % (v/v) inoculum, during a period after the isolation of *S. faecium* repeated subculturing in the urea-free media SY and SBY using a 0.3 % inoculum resulted in cultures which had no urease activity when tested by subculturing into the same media containing urea, i.e. from SY to SU or from SBY to SBU media. From two to six subcultures in either SY or SBY media resulted in loss of activity, and activity was not restored by subsequent repeated, subculturing in urea-containing media. The loss of urease activity was demonstrated in four separate experiments, with triplicate cultures in each, over a period of six to nine months after isolation. However, when these experiments were later repeated, from twelve months to two years after the isolation of the streptococcus, the urease activity was not lost.

Effect of incubation temperature on the synthesis of urease by S. faecium

Since similar results were obtained with both media, only the results from SU medium are quoted in Table 1. At temperatures above 38 °C bacterial growth was reduced, although there is some evidence for an adaptation to the higher temperatures in the third and fourth subcultures. With the higher temperatures urease activity also decreased, but the activity was regained on subculture at 38 °C. At temperatures below 38 °C bacterial growth increased to a maximum between 36 and 29 °C and then decreased. At temperatures below 30 °C urease activity decreased and was permanently lost during culture at 22 or 18 °C.

The effect of SDS on growth and urease activity of S. faecium

SDS was included in SBU medium at concentrations from 0.0005 to 0.0050 % (0.0005 % increments). Up to 0.0010 % SDS, there was no inhibition of growth and all the cultures were ureolytic. After one subculture in 0.0015 % SDS there was a marked reduction in the density (6.8 extinction units compared with 39), but after the second subculture the density had recovered to 37 units. The viable counts (c.f.u./ml) in these cultures were 2×10^7 and 9.8×10^8 , respectively, and 1.48×10^9 in the control. The four cultures grown in 0.0015 % SDS were not ureolytic. However, when any of these cultures was transferred to SDS-free SBU medium, growth and urease activity were regained. At 0.0020 % SDS the growth density in the first subculture, which was not ureolytic, was 4.0 units and the viable count was 7.2×10^6 c.f.u./ml. The density in the second subculture increased to 10.1 units (7.4×10^7 c.f.u./ml). In the fourth subculture with 0.0020 % SDS the density had increased to 13.5 units (1×10^8 c.f.u./ml), but neither culture was ureolytic. The bacteria from the first subculture in the presence of 0.0020 % SDS regained urease activity and growth similar to the control values when transferred to SDS-free medium, but bacteria from the second subculture in the presence of SDS did not regain activity even after 21 successive transfers in SDS-free medium, although growth was similar to the control. The density of the first subculture grown in the presence of 0.0025 % SDS was 3.5 units, and even after three further subcultures at this SDS concentration there was no increase in culture density although there was an apparent marginal increase in viable count – from 6.6×10^6 c.f.u./ml in the first subculture to 7×10^6 in the fourth. One subculture in the presence of 0.0025 % SDS was sufficient to produce permanent loss of urease activity, although the density in the non-ureolytic cultures in SDS-free medium was similar to the control. With SDS at a concentration of 0.0030 % and above there was no visible growth.

Table 1. *Effect of incubation temperature on growth and urease activity of S. faecium*

A stationary-phase culture incubated at 38 °C in SU medium was used to inoculate (0.3 %) 10 ml portions of SU medium which were incubated at different temperatures from 18 to 48 °C (column A). These cultures were successively subcultured three times (columns B, C and D). When there was no urease activity in the fourth culture (column D) grown at a particular temperature then this culture was subcultured into SU medium and incubated at 38 °C (column H). If this culture, grown at 38 °C, was not ureolytic then the experiment was repeated and the first, second and third subcultures were inoculated into SU medium and incubated at 38 °C (columns E, F, G). Growth density was measured at 650 nm and urease activity (i.u.) determined as described in Methods.

Temperature (°C)	Subcultures											
	1			2			3			4		
	A		E	B		F	C		G	D		H
	E_{650}	i.u.	E_{650}	E_{650}	i.u.	E_{650}	E_{650}	i.u.	E_{650}	E_{650}	i.u.	E_{650}
48	9.3	0.01	—	4.3	0	—	13.0	0	—	17.9	0	39.0
46	29.1	0.70	—	28.0	0.58	—	29.0	0.43	—	28.3	0.35	39.1
44	29.2	1.05	—	30.2	0.87	—	31.9	0.76	—	31.2	0.69	39.6
42	33.2	1.00	—	33.9	0.94	—	34.0	1.00	—	33.5	0.96	—
40	37.2	1.19	—	38.0	1.20	—	37.5	1.15	—	37.7	1.18	—
38	39.0	1.21	—	39.2	1.25	—	39.2	1.25	—	39.8	1.20	—
36	41.2	1.28	—	41.0	1.23	—	40.8	1.20	—	41.8	1.23	—
34	41.5	1.24	—	41.2	1.06	—	41.0	0.94	—	41.3	0.92	—
32	41.3	0.99	—	41.0	0.94	—	41.0	0.92	—	41.1	0.88	—
30	41.2	0.84	—	41.0	0.75	—	41.0	0.53	—	41.0	0.48	—
29	41.0	0.56	—	42.0	0.48	—	40.0	0.45	—	43.0	0.24	—
28	39.3	0.26	—	39.8	0.23	—	38.8	0.25	—	39.0	0.19	38.8
27	39.0	0.12	—	38.6	0.03	—	38.0	0	—	39.2	0	38.8
26	38.0	0.08	—	39.0	0	—	39.1	0	—	38.5	0	39.1
25	39.0	0.02	—	38.3	0	—	39.2	0	—	38.9	0	39.0
24	38.0	0.02	—	38.8	0	—	38.5	0	—	39.0	0	39.2
23	39.1	0.01	39.0	39.8	0	38.0	39.0	0	38.7	39.5	0	39.0
22	38.9	0	38.5	38.6	0	38.2	39.0	0	39.0	38.9	0	38.7
21	29.8	0	38.0	30.2	0	37.8	29.8	0	38.4	30.2	0	38.0
20	13.0	0	36.4	15.5	0	36.9	19.1	0	36.8	21.0	0	36.9
18	6.7	0	36.0	6.2	0	36.4	6.0	0	36.3	5.8	0	36.7

The effect of EB on the growth and urease activity of S. faecium

In the first cultures, there was an inhibition of growth with increasing concentrations of EB, with growth ranging from 29.0 units at 9×10^{-6} M (the density of the control was 38 units) to not measurable at 3.0×10^{-5} M and greater concentrations. At 2.5×10^{-5} M-EB, the density was 2.4 units and the viable count 2.6×10^6 c.f.u./ml after one culture. After four subcultures the density had increased to 11.8 units and the viable count to 8×10^7 c.f.u./ml, but there was no measurable urease activity in any culture. However, while ureolytic activity was regained when the first two subcultures with EB were transferred to EB-free medium, when the third subculture was used as inoculum the urease activity was not regained. The action of 3×10^{-5} M-EB was similar. Thus, in the first culture there was no measurable density, although the viable count was 2.6×10^5 c.f.u./ml; after the fourth subculture the density was 9.1 and the count 2.8×10^7 c.f.u./ml. None of the four cultures grown in 3×10^{-5} M-EB were ureolytic although after two subcultures in EB-free medium the bacteria from the first subculture in EB regained the urease activity. Urease activity was permanently lost after the second subculture in medium with EB. There was no measurable growth in the first culture in 3.5×10^{-5} M-EB; the density after four subcultures increased to 8.2 units, and the count increased from 2×10^5 to 2.5×10^7 c.f.u./ml. Urease was lost after two subcultures at 3.5×10^{-5} M-EB.

The effect of EB on the growth and urease activity of P. mirabilis

There was a reduction in culture density from a control value of 30.6 units to 23 and 15.1 after one culture in 6×10^{-5} and 1×10^{-4} M-EB, respectively. Two further subcultures in 1×10^{-4} M-EB resulted in a slight increase in culture density from 15.1 to 20 units. The density in the first culture in 2×10^{-4} M-EB was zero but in the second culture was 16.4 units. An EB concentration of 3×10^{-4} M was completely inhibitory; there was no measurable growth in successive subcultures and no colonies were formed when these cultures were plated on brain-heart infusion agar. All the cultures grown in the presence of 1×10^{-4} M-EB were ureolytic. In 2×10^{-4} M-EB the urease activity of the first culture was not measurable because of insufficient growth, but 100 colonies isolated from this culture were all ureolytic. In the succeeding subcultures grown in 2×10^{-4} M-EB, urease could be measured but it was only about half that of the control.

Effect of EB on growth and urease activity of P. provostii

The culture density in medium AE without EB was 80 units and the effect of increasing concentrations of EB was to inhibit growth, until at 4.5×10^{-6} M there was no measurable growth. At 4×10^{-6} M the culture density was 1.7 units and urease activity was very small, while at 3.5×10^{-6} M the density was 21.9 units and the culture was definitely ureolytic. Further subcultures in the presence of EB at 4.5×10^{-6} M did not result in any increase in growth, but with 4×10^{-6} M the culture density increased to 13.8, 26.9 and 81 units in successive subcultures, while in the presence of 3.5×10^{-6} M-EB the density increased to 72 units after two subcultures. Urease activity could be measured in all subcultures in either 4×10^{-6} or 3.5×10^{-6} M-EB, and all the 100 colonies isolated from the culture incubated once in the presence of EB at 4×10^{-6} M were positive.

Effect of aeration on urease activity of S. faecium

Bacteria grown for 24 h under aerobic conditions are not ureolytic and activity is not regained in subsequent anaerobic subcultures (Cook, 1976). The present experiments tested

Table 2. *Effect of aeration on growth density and urease activity of S. faecium*

The growth density (E_{650}) and urease activity (i.u.) of an aerobic culture of *S. faecium* grown in SBU medium (105 ml) at 38 °C without sodium thioglycollate and aerated at 3000 ml/min. Anaerobic SBU medium was inoculated at 0.5 h intervals with inocula from the aerobic culture and grown for either 24 or 48 h at 38 °C. The anaerobic cultures grown for 24 h were subcultured into SBU medium and incubated a further 24 h. The growth density was measured at 650 nm and urease activity was determined for each subculture. Urease activity of the aerobic culture was determined as described in Methods; in the subculture, 1 ml of culture was resuspended after centrifugation, in 1 ml of the assay buffer, and a 0.2 ml sample assayed overnight. The result was scored as positive (+) or negative (—).

Time (h)	Anaerobic subcultures							
	Aerobic culture		First 24 h		48 h		Second 24 h	
	E_{650}	i.u.	E_{650}	Urease	E_{650}	Urease	E_{650}	Urease
0	0	0.0012	42.5	+	29.1		50.0	
0.5	0.5	0.0012	38.8	+	30.2		47.7	
1.0	1.3	0.0011	39.0	+	31.1		47.8	
1.5	2.3	0.0012	40.2	+	30.9		46.0	
2.0	2.8	0.0011	40.0	+	31.2		47.0	
2.5	3.6	0.0010	38.0	+	28.8		46.8	
3.0	4.9	0.0011	36.8	+	27.4		46.8	
3.5	8.3	0.0011	31.3	+	23.0		46.8	
4.0	15.3	0.0010	26.8	+	16.7		44.2	+
4.5	22.8	0.0009	20.1	—	13.5	+	43.8	+
5.0	28.8	0.0006	18.6	—	8.8	—	41.5	—
5.5	32.8	0.0006	16.0	—	6.4	—	44.2	—
6.0	43.8	0.0005	14.9	—	5.1	—	42.9	—
6.5	44.8	0.0004	14.0	—	5.0	—	42.1	—
7.0	47.8	0.0004	13.0	—	4.8	—	42.5	—
7.5	48.8	0.0002	12.5	—	5.0	—	42.3	—
8.0	48.5	0.0002	13.2	—	5.2	—	42.0	—
8.5	48.7	0.0001	13.0	—	5.0	—	42.4	—
9.0	48.3	0.0000	12.8	—	4.8	—	42.1	—

the effects of aeration in more detail. After 5 h aeration urease activity was permanently lost, as it could not be restored by continued anaerobic subculture (Table 2).

Isolation of plasmid DNA (CCC) by dye-buoyant density centrifugation

Examination of the centrifuge tubes after density centrifugation showed the presence of only one band, equivalent to that present in the centrifugate of DNA obtained from urease-negative bacteria treated with EB.

DISCUSSION

Conjugation ability, production of colicins, and some (but not all) resistance to antibiotics and inorganic ions appear to be coded only on extrachromosomal genetic determinants (plasmids), but there is now increasing evidence that there can be other plasmid-coded bacterial characters.

Richmond (1972) listed five criteria for the identification of the extrachromosomal location of genes, and the first of these criteria refers to the high frequency of loss. It was observed during the isolation of *S. faecium* that urease activity was not present in all the bacteria. Thus the streptococcus was usually isolated without urease activity from dilutions of either the rumen or enrichment cultures, and even most of the colonies isolated from a

diluted, ureolytic, pure culture of *S. faecium* were not ureolytic. It was thus tentatively suggested (Cook, 1976) that the genetic coding for urease might be sited on a plasmid. Urease activity was retained in the mixed enrichment subcultures made before the isolation of the streptococcus in pure culture, but a large inoculum (1.5 %, v/v) was used for these. In the mixed continuous culture system of the normal rumen the streptococcus appears to produce most of the relatively constant urease activity, and a gnotobiotic lamb inoculated with the streptococcus and other bacteria had a stable rumen-urease level (Cook, 1976).

The first criterion of Richmond (1972) states that the loss frequency should be quantified, to differentiate the high frequency of loss associated with plasmids from that of the spontaneous mutation rates of most genes. With the urease activity of *S. faecium* it has not been possible to evaluate such a figure, since virtually all the colonies isolated from a diluted pure culture were non-ureolytic and so only an 'all-or-nothing' effect could be obtained.

Another criterion listed by Richmond (1972) is the effect of growth at elevated temperatures on the spontaneous rate of loss of a plasmid-borne property (Fairbrother, Parker & Eaton, 1954). With *S. faecium*, while there was no measurable urease activity in a culture incubated at 48 °C and thus the rate of spontaneous loss was increased at this temperature, activity returned after subculture at 38 °C, which is both the usual culture temperature and the temperature of the rumen. Lowering the incubation temperature, however, presumably caused a greater rate of loss, resulting in a permanent deletion of urease activity at 22 °C and below.

The effect of chemical agents on the spontaneous rate of loss of a genetic marker is a further criterion of Richmond (1972). The chemical agents used in this study were (i) SDS which may act by disrupting the plasmid-membrane attachment site (Sonstein & Baldwin, 1972*b*), and (ii) the trypanocidal drug EB, which, like the acridine dyes, intercalates between base pairs of DNA, thus indirectly hindering the action of DNA and RNA polymerases (Waring, 1966). With *S. faecium*, one subculture in the presence of 0.0025 % SDS was sufficient to 'cure' the bacterium of ureolytic activity, and with SDS at 0.0020% two subcultures were needed to obtain a non-ureolytic culture.

The effect of EB on the elimination of urease activity from *S. faecium* was compared with the effect of this 'curing' agent on the urease activity of *Proteus mirabilis* and *Peptostreptococcus provostii*. *Streptococcus faecium* was 'cured' of urease activity when subcultured three times in the presence of EB at 2.5×10^{-5} M, which was less than the minimal inhibitory concentration of EB at 3×10^{-5} M. On the other hand *P. mirabilis* and *Pep. provostii* still retained urease activity even at EB concentrations at which there was no measurable growth. The results of Bouanchaud, Scavizzi & Chabbert (1969) with five strains of *Staphylococcus aureus* support the present results with *S. faecium* in that they found that 5×10^{-6} to 10×10^{-6} M-EB eliminated penicillin resistance in the staphylococcus. Similarly Sonstein & Baldwin (1972*a*) found the highest rate of elimination of plasmid-coded penicillin resistance in *Staph. aureus* was with 3×10^{-5} M-EB, and Courvalin *et al.* (1972) 'cured' *Streptococcus faecalis* of tetracycline resistance with EB at 2.5×10^{-5} M.

Thus the actions of SDS and EB, and the growth conditions of low temperature and aeration, resulted in the loss of urease activity from *S. faecium*, and it is proposed that these results are evidence for a plasmid-coded urease. The inability to isolate a plasmid from the *S. faecium* is not, of course, necessarily proof of its non-existence. If, in fact, only about 1 % of the cells in a culture are urease-positive then sufficient CCC DNA for detection by ultra-violet examination might not be present. This possibility is being investigated.

Another possible explanation is that the loss of urease activity may be the result of one of two kinds of chromosomal mutation. In the first, a mutator gene may be present which

greatly enhances the frequency of point mutations of certain sites in permanently chromosomal genes (Richmond, 1972). In the second, the insertion of a few nucleotide pairs into the continuity of a chromosomal gene (e.g. urease) would result in a spontaneous mutation (Jordan, Saedler & Starlinger, 1968). Saedler & Heib (1973) proposed that a small plasmid may fulfil this role of carrying an insertion sequence, but were not able to detect such.

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