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Induction of Autolysis in *Streptococcus faecium*

By M. EGIDIA CARVALHO,* M. HELENA GONÇALVES
AND MANUEL T. SILVA

*Instituto de Ciências Biomédicas 'Abel Salazar', Largo da Escola Médica, 4000 Porto
and Centro de Citologia Experimental da Universidade do Porto, Porto, Portugal*

Autolysis of exponential-phase *Streptococcus faecium* cells was promoted by pretreating the bacteria (freezing–thawing; $-70\,^\circ$C) in Tris buffer, followed by incubation at $37\,^\circ$C in the same buffer. The effect was dependent on Tris concentration. The pretreatment provoked ultrastructurally visible damage with extensive loss of K^+ and leakage of UV-absorbing components. No autolysis was observed when the bacteria frozen–thawed in Tris were incubated in the presence of the autolysin inhibitor *N*-bromosuccinimide nor when they had been grown in the presence of chloramphenicol or tetracycline. Furthermore, two autolytic-defective mutants, EC31 and EC78, isolated from *S. faecium*, did not autolyse when frozen–thawed and incubated in Tris. Freezing–thawing in Tris, however, imparted extensive cell damage to the mutants and to the antibiotic-treated bacteria as well as considerable leakage of K^+ and UV-absorbing materials. These observations indicate that the lysis of *S. faecium* reported above is due to the activity of the endogenous bacterial autolysin. Induction of autolysis of *S. faecium* by freezing–thawing was also observed, although to a lesser extent, when Tris was replaced by imidazole.

INTRODUCTION

Bacterial autolysins are generally found in exponential-phase cultures but to a lesser extent in stationary-phase cultures, suggesting a role in the overall process of bacterial growth (Rogers, 1979; Shockman *et al.*, 1967). Bacterial autolysins have been implicated in many important biological phenomena (Pooley, 1976; Ranhand *et al.*, 1971; Rogers, 1979; Pooley & Karamata, 1984). Several factors influence the occurrence and extent of bacterial autolysis including growth conditions, osmotic environment and pH of the medium (Shockman, 1965; Carson & Daneo-Moore, 1980).

Streptococcus faecium ATCC 9790 generally does not lyse spontaneously, some kind of induction being necessary to bring about rapid and extensive autolysis. We have previously shown that fresh *S. faecium* cells do not lyse either in phosphate or Tris buffer (pH 8·0); however, when they are frozen–thawed ($-70\,^\circ$C) in Tris buffer before incubation in the same buffer some autolysis is obtained (Carvalho *et al.*, 1984). This observation prompted us to study in more detail the induction of autolysis by the freezing–thawing of cells in Tris, imidazole and phosphate buffers at the optimum pH for the activity of *S. faecium* autolysin (pH 6·8) (Shockman *et al.*, 1967; Sayare *et al.*, 1972).

METHODS

Bacterial strains. Most of the experiments were done with *Streptococcus faecium* ATCC 9790. Two mutants, EC31 and EC78, were isolated from *S. faecium* by mutation with ethylmethane sulphonate and characterized as autolytic-defective as described by Pooley *et al.* (1972).

Abbreviations: CAM, chloramphenicol; TC, tetracycline; NBS, *N*-bromosuccinimide.

Growth conditions. The wild-type strain of *S. faecium* and the two autolytic-defective mutants were grown at 37 °C in medium T [1%, w/v, tryptone (Difco); 0·5%, w/v, yeast extract (Difco); 0·25%, w/v, K_2HPO_4; 1%, w/v, glucose, pH 7·2] (Carvalho *et al.*, 1984). In some experiments, 30 min before the harvest of cells, chloramphenicol (CAM; 10 µg ml^{-1}; Sigma) or tetracycline (TC; 0·5 µg ml^{-1}; Sigma) was added to the cultures. Growth was followed by measuring the OD_{520} in a Bausch & Lomb Spectronic 20 spectrophotometer.

Autolysis assays. Early exponential-phase cells (OD_{520} 0·5) were harvested by centrifugation for 10 min at 15 000 *g*, and washed and resuspended in the appropriate lysis buffers or in medium T without K_2HPO_4. Some samples were frozen as concentrated suspensions at −70 °C in a deep-freezer before use. Suspensions (20 ml; 7·2 × 10^8 cells ml^{-1}) of freshly grown cells or frozen cells thawed at room temperature were transferred to 250 ml flasks and incubated for 120 min at 37 °C in the following buffers: 0·01–0·5 M-Tris/HCl, pH 6·8; 0·01–0·4 M-imidazole (grade III, Sigma)/HCl, pH 6·8; 0·01–0·5 M-NaH_2PO_4/Na_2HPO_4, pH 6·8; 0·01–0·5 M-Na_2HPO_4/KH_2PO_4, pH 6·8. Where indicated, *N*-bromosuccinimide (NBS, Sigma) was added to the lysis buffer at a final concentration of 1 mM. In some experiments, $MgSO_4$ was added to the lysis buffer at a final concentration of 10 or 100 mM. In all cases, the optical density of the suspensions was followed by a Bausch & Lomb Spectronic 710 spectrophotometer.

K^+ efflux assays. The efflux of K^+ was determined by assaying filtrates obtained in less than 30 s with membrane filters (type HA, pore size 0·45 µm, Millipore Corp.). The values are given as a percentage: a 100% value corresponds to the K^+ leaked from bacteria boiled for 30 min. K^+ was assayed with an EEL flame photometer, model 150.

Leakage of UV-absorbing materials. Using filtrates obtained as indicated above, the amount of intracellular material that absorbs at 260 and 280 nm, respectively, was determined with a UV-visible recording spectrophotometer (UV-260 Shimadzu) in samples of fresh or frozen–thawed bacterial suspensions in various buffers as indicated below.

Electron microscopy. Bacteria for ultrastructural studies were collected both before incubation in the autolysis buffers and at intervals during incubation. In both cases fresh and frozen–thawed cells were studied. Fixation was by adding stock 25% (w/v) glutaraldehyde (TAAB Laboratories) directly to the samples to a final concentration of 2·5% (w/v). In some experiments, frozen cells were thawed, centrifuged and resuspended in 0·05 M-sodium cacodylate buffer, pH 7·0, and then fixed with glutaraldehyde as described above. After 60 min, bacteria were pelleted by centrifugation and post-fixed with 1·0% (w/v) OsO_4 (Ryter & Kellenberger, 1958) followed 16 h later by 1·0% (w/v) aqueous uranyl acetate (Silva *et al.*, 1971). The fixed pellets were processed for ultramicrotomy by embedding in Epon 812 (Luft, 1961). The ultrathin sections were contrasted with uranyl acetate (aqueous saturated solution) for 50 s followed by lead citrate (Venable & Coggeshall, 1965) for 2 min; they were observed under a Siemens Elmiskop 1A transmission electron microscope.

Characterization of membranes isolated from autolysed S. faecium cells. Membrane vesicles were isolated from *S. faecium* suspensions that had been allowed to autolyse until a constant OD_{520} value was reached. The final lysate was centrifuged at 800 *g* for 10 min to separate cell debris, and the supernatant was spun at 30 000 *g* for 30 min to pellet the membrane vesicles. These vesicles were washed once in the autolysis buffer. The protein content of the membrane vesicles was assayed by the Lowry method, using lysozyme (grade 1, Sigma) as standard. The SDS-PAGE protein profile of the isolated membranes was as described by Carvalho *et al.* (1984). The ultrastructure of these membranes was studied by the methods indicated above. Tracings of membrane profiles were made on photographic negatives with a Joyce-Loebl Mk. III CS microdensitometer, set to an arm ratio of 50 × and a slit width of 0·5 nm.

RESULTS

Effect of freezing–thawing in different buffers on S. faecium autolysis

When exponential-phase *S. faecium* cells were suspended in 0·01–0·5 M-Tris buffer, no lysis was observed during 120 min of incubation (Fig. 1), and the ultrastructural study showed unaltered cells. However, lysis was efficiently promoted when bacteria were frozen and thawed in Tris buffer before incubation (Fig. 1). As shown below, the lysis was due to the activity of the *S. faecium* autolysin.

The rate of autolysis of frozen cells was dependent on Tris concentration and was maximal at 0·1 M (Fig. 1). Samples for electron microscopy collected immediately after the freezing–thawing of bacteria in 0·1 M-Tris buffer showed damaged bacteria (Fig. 2*a*). Such damage is due to the direct effect of Tris on bacteria, since similar ultrastructural alterations were seen when bacteria frozen in Tris buffer were then fixed with glutaraldehyde in cacodylate (not shown). On the other hand, the K^+ efflux from *S. faecium* cells frozen in 0·1 M-Tris buffer was much increased when compared with the K^+ efflux from fresh bacteria (Fig. 3).
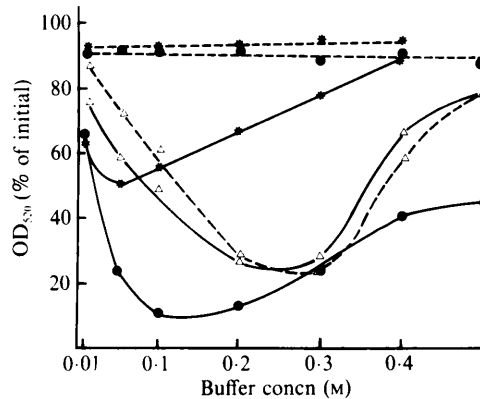
Fig. 1. Effect of freezing–thawing and of several buffers on the autolysis of *S. faecium*. Exponential-phase cells, fresh (– – – –) or previously frozen–thawed (———), were suspended in sodium phosphate, Tris or imidazole buffers, pH 6·8. Autolysis was assayed by the loss of optical density of cell suspensions ($7·2 \times 10^8$ cells ml^{-1}) during 120 min incubation at 37 °C. $\triangle$, Sodium phosphate buffer; ●, Tris/HCl buffer, *, imidazole/HCl buffer.

Table 1. *Cellular autolysis of S. faecium*

Exponential-phase cells were washed and frozen–thawed in the indicated buffers or in medium T without K_2HPO_4. Where indicated, after thawing the bacteria were centrifuged at 15000 *g* for 5 min to remove the buffers used for freezing–thawing and resuspended in the incubation buffers. Autolysis was assayed by the decrease in optical density of cell suspensions ($7·2 \times 10^8$ bacteria ml^{-1}) during 120 min incubation at 37 °C. The results are expressed as a percentage of the initial optical density. ND, Not determined.

	Incubation buffer		
Freezing–thawing buffer	Tris (0·1 M)	Sodium/potassium phosphate (0·01 M)	(0·3 M)
Tris (0·1 M)	11	18	41
Sodium/potassium phosphate (0·01 M)	64	78	43
(0·3 M)	40	28	41
Medium T	90	ND	41
No freezing–thawing	90	86	23

Imidazole buffer yielded results similar to those obtained with Tris (Fig. 1). However, the rate of autolysis of bacteria frozen in imidazole buffer was lower than in Tris buffer and was maximal at a concentration of 0·05 M. Ultrathin sections of cells collected immediately after freezing–thawing in 0·05 M- or 0·4 M-imidazole buffer also showed damaged bacteria, and for 0·05 M buffer the K$^+$ efflux was much increased when compared with the K$^+$ efflux from unfrozen bacteria suspended in the same buffer (Fig. 3). Cells frozen–thawed in 0·4 M-Tris or imidazole buffers lost all their intracellular K$^+$ (not shown).

Additional experiments (Table 1) showed that: (i) freezing–thawing in 0·1 M-Tris resulted in activation of the *S. faecium* autolysin, especially when the cells were incubated in 0·1 M-Tris or in 0·01 M-sodium/potassium phosphate; (ii) previous freezing–thawing in phosphate buffer does not increase the rate of autolysis of fresh bacteria in the same buffer (Fig. 1); (iii) freezing–thawing in broth does not promote the activation of the autolysin, since no lysis occurred when cells so treated were incubated in 0·1 M-Tris buffer. Interestingly, freezing–thawing in phosphate (Fig. 2*b*) or broth did not induce ultrastructural alterations (not shown) and resulted in a moderate leakage of K$^+$ (Fig. 3) and UV-absorbing material (not shown).
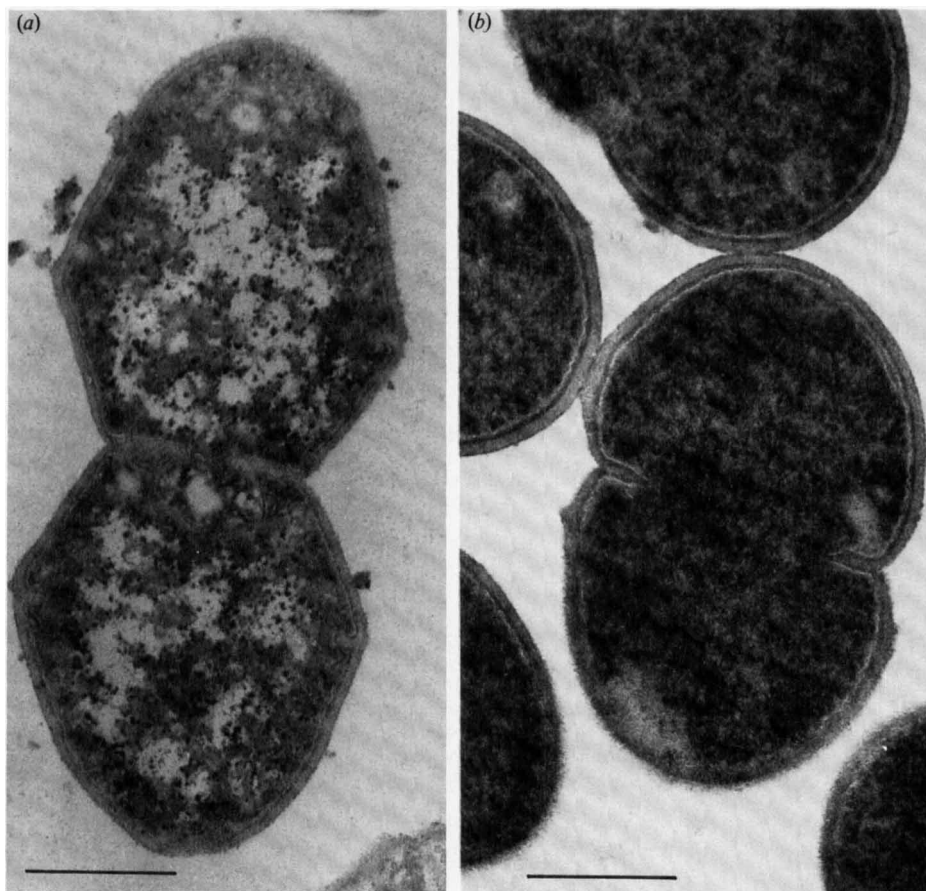
Fig. 2. Ultrastructure of *S. faecium* collected immediately after freezing–thawing (−70 °C) in (*a*) 0·1 M-Tris buffer, pH 6·8 – note the altered cell contents and absence of ribosomes – or (*b*) 0·3 M-sodium phosphate buffer, pH 6·8 – note that cells show a normal ultrastructure, namely intact cell walls and ribosomes. Bars, 400 nm.

Role of S. faecium autolysin in the lytic process

The exposure of exponential-phase cultures of *S. faecium* to CAM (10 µg ml⁻¹) or TC (0·5 µg ml⁻¹) extensively inhibited autolysis of fresh or frozen–thawed bacteria. Freezing–thawing of CAM-treated cells suspended in Tris resulted, however, in ultrastructural damage similar to that with untreated cells, and in extensive leakage of K⁺ and UV-absorbing materials (not shown). Moreover NBS, when added at final concentration of 1 mM, completely inhibited cellular autolysis of cells in Tris buffer (not shown).

Two autolytic-defective mutants of *S. faecium*, EC31 and EC78, showed a much-decreased capacity for cellular autolysis after freezing–thawing in Tris, a treatment that resulted in extensive cell damage (Fig. 4) and significant efflux of K⁺ and UV-absorbing materials (not shown).

Influence of Mg²⁺

The presence of 10 mM-Mg²⁺ in 0·1 M-Tris buffer during freezing–thawing and incubation partially decreased the rate and extent of autolysis of frozen cells, as well as the leakage of UV-absorbing materials, but did not prevent the high K⁺ efflux (Fig. 3). However, 10 mM-Mg²⁺ prevented in part the cytoplasmic membrane damage since after 120 min incubation intact
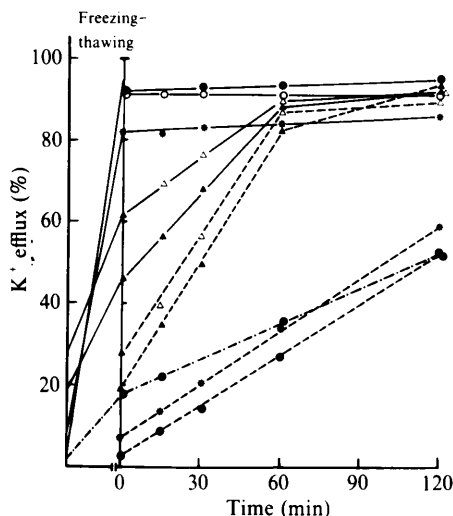
Fig. 3. K^+ efflux after freezing–thawing and/or during incubation of *S. faecium* in various conditions. Unfrozen cells (—---) or cells frozen ($-70\ °C$) in Tris, phosphate or imidazole buffers (——) or in medium T without K_2HPO_4 (—·—·) were resuspended (7.2×10^8 cells ml^{-1}) and incubated at 37 °C in Tris, phosphate or imidazole buffers. Filtrates of samples collected after freezing–thawing or during incubation were assayed for K^+. The K^+ content of filtrates of each suspension boiled for 30 min was normalized to 100%. ●, 0·1 M-Tris; ○, 0·1 M-Tris and 10 mM-MgSO₄; ∗, 0·05 M imidazole; ▲, 0·2 M, △, 0·3 M-sodium phosphate buffer.

protoplasts were visible (not shown). Furthermore, autolysis was strongly inhibited when 100 mM-Mg^{2+} was added at the beginning of the autolytic process, or partially inhibited when it was added 30 min later to the autolysing cells [in the first case the final OD_{520} value was 74%, and in the second case 57%, of the initial value, instead of 11% when Mg^{2+} was not present (Fig. 1)]; in both cases the ultrastructural studies, in accordance with the optical density data, revealed intact protoplasts and many unlysed cells, some of them with normal cell walls (not shown).

Characteristics of membranes isolated in Tris or phosphate buffers

Thin sections of membranes isolated from bacteria frozen and incubated in 0·05 M-Tris buffer (Fig. 5a) showed a loose triple-layered structure with a thickness of 5·68 ± 0·43 nm (peak-to-peak distance in microdensitometric tracings). Membranes isolated from bacteria frozen and incubated in 0·02 M sodium/potassium phosphate showed well defined triple-layered profiles, with a thickness of 6·25 ± 0·83 nm (Fig. 5b). Furthermore, 30% of membrane protein was lost from membranes isolated from *S. faecium* cells frozen and incubated in 0·05 M-Tris buffer, as compared to the membranes isolated from cells frozen and incubated in 0·02 M-phosphate buffer. The protein fractionation by SDS-PAGE of the two types of isolated membranes showed (Fig. 6) that the membranes isolated by autolysis of *S. faecium* cells frozen–thawed and incubated in Tris had a decreased content of proteins with M_r values above 30000. A similar result was obtained with imidazole buffer (not shown).

DISCUSSION

In the present paper we describe lysis of *S. faecium* cells as assessed by spectrophotometry and electron microscopy. The participation of *S. faecium* autolysin was inferred from the following data. (i) Cultivation of *S. faecium* in the presence of several inhibitors of protein synthesis prevents the expression of its endogenous autolytic activity (Sayare *et al.*, 1972). In the present work we found that pre-exposure of *S. faecium* to two such inhibitors (CAM and TC) prevented

Fig. 4. Ultrastructure of mutant EC78 immediately after freezing–thawing in 0·05 M-Tris buffer, pH 6·8. Note the altered membranes and cell contents with absence of ribosomes. Bar, 300 nm.

the extensive lysis that occurred with frozen cells suspended in Tris buffer. (ii) The autolytic activity of S. faecium can also be stopped by NBS, a molecule that promotes direct inactivation of the autolysin (Ramachandran & Witkop, 1967; Cornett & Shockman, 1978); such a treatment resulted in complete inhibition of the lysis of S. faecium frozen–thawed in Tris buffer. (iii) The use of autolytic defective-mutants derived from S. faecium (EC31 and EC78) provided additional evidence in favour of the role of the autolysin in the autolytic process under discussion; both mutants showed much-reduced autolysis when frozen and incubated in 0·05 M-Tris buffer, conditions that induce extensive autolysis of the wild-type strain.

We showed previously (Carvalho et al., 1984) that autolysis of S. faecium occurs in Tris buffer only when the cells are frozen–thawed before incubation. Now, we found that freezing–thawing of cells in Tris buffer efficiently promoted autolysis when the cells were incubated in the same buffer used for freezing–thawing. Additionally, freezing–thawing of S. faecium in the Tris buffers resulted in extensive K^+ efflux and leakage of UV-absorbing material, and in major ultrastructural changes, including absence of ribosomes and altered membranes. Such signs of membrane damage were not observed when the bacteria were suspended in Tris buffers at room temperature. That Tris damages S. faecium membranes is also shown by the observation that membranes isolated after lysis of bacteria frozen and incubated in Tris buffer have an altered ultrastructure, a 30% decrease in protein content and are thinner in comparison with membranes isolated from bacteria frozen in phosphate buffer. Freezing–thawing of S. faecium in phosphate buffer does not result in increased autolysis, and is not accompanied by significant ultrastructural damage or efflux of K^+ and UV-absorbing materials.

The mechanism by which Tris buffer, at very low temperature, induces the expression of S. faecium cellular autolytic activity, is not presently known. Although alterations induced by freezing–thawing in the cell wall (which, in turn, would result in the uncontrolled action of the autolysin by a yet unknown mechanism) or the promotion of an alteration in the autolytic enzyme itself (for example by activating a latent form) could be involved, our data suggest than activation of S. faecium autolysin is secondary to the damage to the cytoplasmic membrane inflicted by freezing–thawing in Tris. Tris is not physiologically inert (Voss, 1967): for example, it increases the outer-membrane permeability of Escherichia coli by disrupting ionic interactions (Irvin et al., 1981; Schindler & Teuber, 1978). Moreover, Alonso et al. (1979) obtained results which suggest that Tris causes an increase in the permeability of, or damage to, sarcoplasmic reticulum membranes, and found that such alterations were reproduced by replacing Tris with imidazole; their interpretation was that both molecules act through similar mechanisms. We
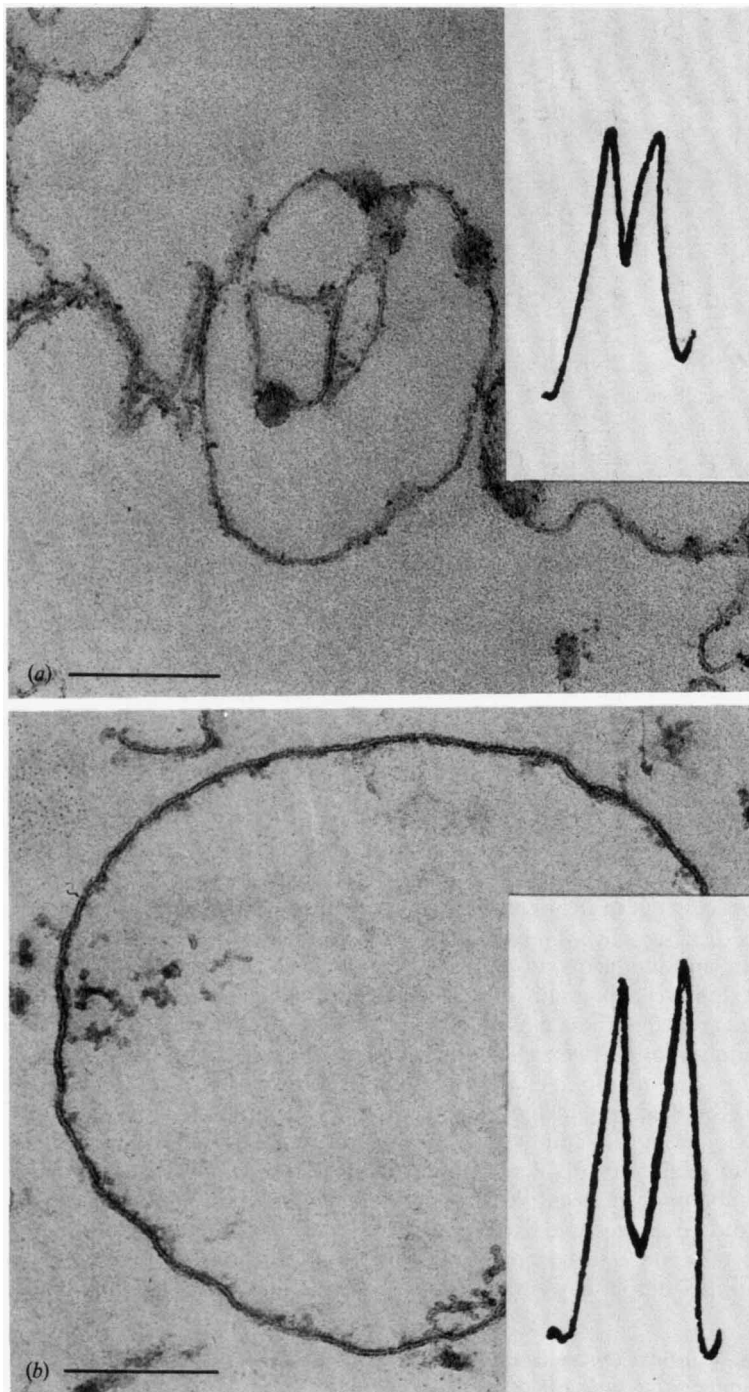
Fig. 5. Ultrastructure of membranes isolated from *S. faecium*. (*a*) Membranes isolated after autolysis in 0·05 M-Tris buffer, pH 6·8. Cells had previously been frozen–thawed in the same buffer. (*b*) Membranes isolated after autolysis in 0·02 M-sodium/potassium phosphate buffer, pH 6·8, after freezing–thawing in the same buffer. Note the loose ultrastructure of the membranes isolated in Tris buffer (*a*) in contrast with membranes isolated in phosphate buffer (*b*). Bars, 270 nm. Inset: densitometric tracings of membrane profiles.
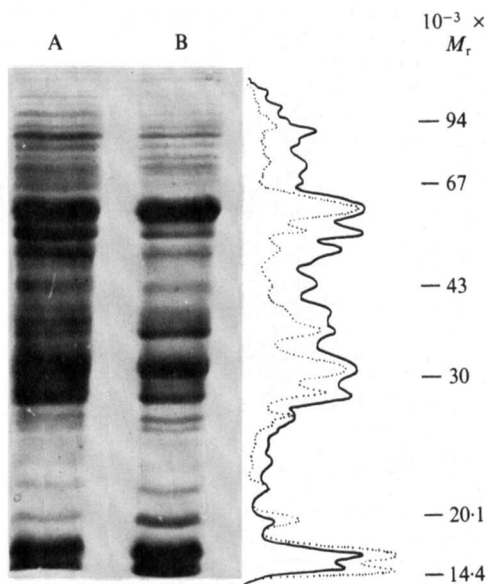
Fig. 6. SDS-PAGE of proteins from membranes isolated from *S. faecium* by autolysis. Lane A, membranes from bacteria frozen–thawed and incubated in 0·02 M-sodium/potassium phosphate buffer; lane B, membranes from bacteria frozen–thawed and incubated in 0·02 M-Tris buffer. In A and B the samples contained membrane protein obtained from an equal number of bacteria. The traces represent the bands in A (———) and B (······). Note that the higher amount of protein in the membranes obtained by autolysis in phosphate buffer as compared to that in the membranes obtained by autolysis in Tris buffer is mainly due to the increase in the amounts of proteins with M_r values above 30000.

therefore compared the effects of imidazole with those of Tris. Imidazole also promoted lysis of *S. faecium* by freezing–thawing, and induced ultrastructural alterations, extensive K^+ efflux and significant leakage of UV-absorbing material. The effects ascribed to Tris in the present work are thus not specific but rather depend on physico-chemical characteristics common to Tris and imidazole, as discussed by Alonso *et al.* (1979). In contrast to these reports of the damaging actions of Tris and imidazole, in our study the alterations to *S. faecium* cells, that eventually resulted in extensive expression of endogenous autolytic activity, occurred only when the bacteria were exposed to these molecules during freezing–thawing. Jolliffe & Doyle (1981) have shown that autolysis of *Bacillus subtilis* can be induced by treatments that disrupt electrical or pH gradients across the membrane, a mechanism that was considered by those authors to be applicable to other bacteria, including *S. faecium*. It is possible that freezing–thawing in Tris or imidazole is triggering the autolytic activity in our model through that mechanism.

The inhibitory effect of divalent cations on the expression of autolytic activity has been observed with both Gram-negative (Rayman & MaCleod, 1975; Leduc *et al.*, 1982; Parente & Silva, 1984) and Gram-positive bacteria (J. Poiares & M. T. Silva, unpublished results with *B. subtilis*). Our results are in accord in showing that addition of 10 mM-Mg^{2+} to Tris buffer partially inhibited autolysis of *S. faecium* and gave some stabilization to the cytoplasmic membrane.

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