

The Involvement of the Proteinase of *Streptococcus faecium* ATCC 9790 in the Stimulation of Its Autolysin Activity by Dodecylglycerol*

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H. S. Ved, Evan Gustow, and Ronald A. Pieringer

From the Department of Biochemistry, Temple University School of Medicine, Philadelphia, Pennsylvania 19140

Treatment of *Streptococcus faecium* ATCC 9790 with 3.5 $\mu\text{g/ml}$ of dodecylglycerol produces a nonwall entity found in the 25,000 $\times g$ supernatant cell fraction which activates the autolysin activity of *S. faecium*. The stimulation of the autolysin activity by dodecylglycerol mimics the activation of the autolysin from a latent to an active form by trypsin and other proteolytic enzymes. This stimulation of autolytic activity by dodecylglycerol can be reversed by specific proteinase inhibitors. Dodecylglycerol also markedly stimulates the proteinase activity endogenous to *S. faecium*, and this stimulation can be reversed by several proteinase inhibitors. It is concluded that one primary antibacterial mode of action of dodecylglycerol is to stimulate the proteinase of *S. faecium* which activates the cell's autolysin and thereby prevents bacterial growth.

trichloroacetic acid. After filtration, the absorbance of the soluble fraction was measured at 280 nm. Control assays were carried out as above except dodecylglycerol was omitted.

Other Methods—All other methods including culture conditions, autolysin, assays, and isolation of cell walls, were carried out exactly as described in the accompanying paper (1).

RESULTS

Cell Wall Autolysis Stimulated by 25,000 $\times g$ Supernatant from Dodecylglycerol-treated Cells—Previous experiments (1) showed that dodecylglycerol added directly to isolated cell wall preparations (25,000 $\times g$ pellet) did not stimulate the autolysin activity in these preparations. However, the 25,000 $\times g$ supernatant from broken cells of *S. faecium* previously grown in the presence of a sublethal dose (3.5 $\mu\text{g/ml}$) of dodecylglycerol stimulated cell wall autolysin activity to a

In the accompanying paper (1), dodecylglycerol was shown to inhibit the growth of *Streptococcus faecium* ATCC 9790 at relatively low concentrations. Because it also readily stimulated the autolysin activity of whole cells of *S. faecium* ATCC 9790 at the same low concentrations, this stimulation of the cell's autolysin was suggested as one possible mode of antibacterial action by dodecylglycerol. However, the autolysin activity of isolated cell walls, where most autolysin activity of *S. faecium* resides (2), could not be stimulated by dodecylglycerol. These data suggest that an intermediary of membrane or cytosolic origin is required to stimulate the autolysin of the wall by dodecylglycerol. One possible candidate that might function as an intermediary is a proteinase since the autolysin of *S. faecium* is known to be converted from an inactive, latent form to an active enzyme by proteolytic action (3). The results of experiments which test this hypothesis are presented here.

EXPERIMENTAL PROCEDURES

Proteinase Activity in *S. faecium*—The following assay was adopted from Kunitz (4). *S. faecium* was grown in 30 ml of chemically defined medium (5) to midlog phase (~ 0.5 adjusted optical density) and harvested by centrifugation. After washing, the cells were suspended in 5 ml of sodium phosphate buffer (0.1 M, pH 7.6). The cells were treated with dodecylglycerol at a concentration of 20 $\mu\text{g/ml}$ for 10 min at 37 $^{\circ}\text{C}$ and then were disrupted by sonication at 0–4 $^{\circ}\text{C}$ until the turbidity was reduced to one-third. One-half ml of this suspension was used in each assay for proteolytic activity. Proteinase inhibitors were added and the reaction was initiated by the addition of 1.0 ml of 0.1% casein in 0.1 M sodium phosphate buffer (pH 7.6). The reaction was terminated after 30 min at 37 $^{\circ}\text{C}$ by the addition of 10%

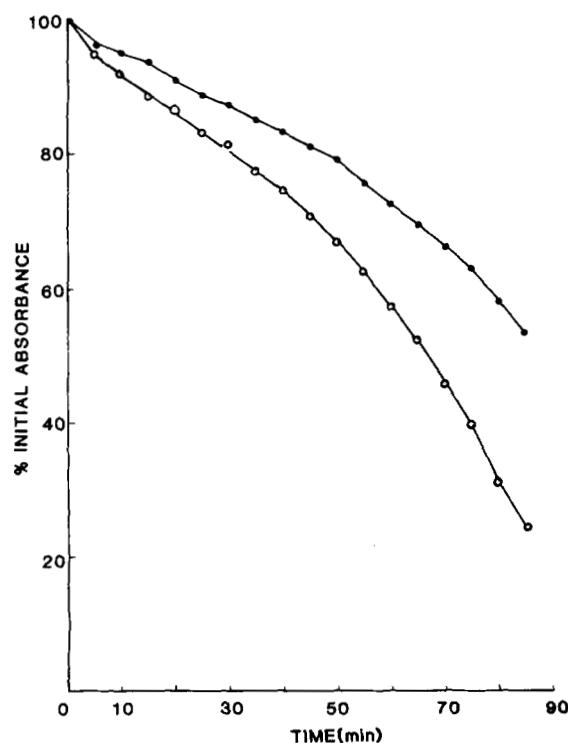


FIG. 1. Cell wall lysis: effect of 25,000 $\times g$ supernatant from dodecylglycerol-treated cells. *S. faecium* cells were grown up to an OD_{675} of 0.05, treated with either zero (control) (●) or 3.5 $\mu\text{g/ml}$ of dodecylglycerol (○), and harvested when control reached an OD_{675} of 0.5–0.6. The cells were washed three times with cold distilled water. Washed cells were suspended in phosphate buffer (0.01 M, pH 6.7) sonicated, and centrifuged at 25,000 $\times g$ for 15 min (0–4 $^{\circ}\text{C}$). Supernatant (1.5 ml) was added to *S. faecium* cell wall suspension (6 mg/10 ml of phosphate buffer), and the lysis of the cell wall was followed spectrophotometrically at 475 nm and 37 $^{\circ}\text{C}$.

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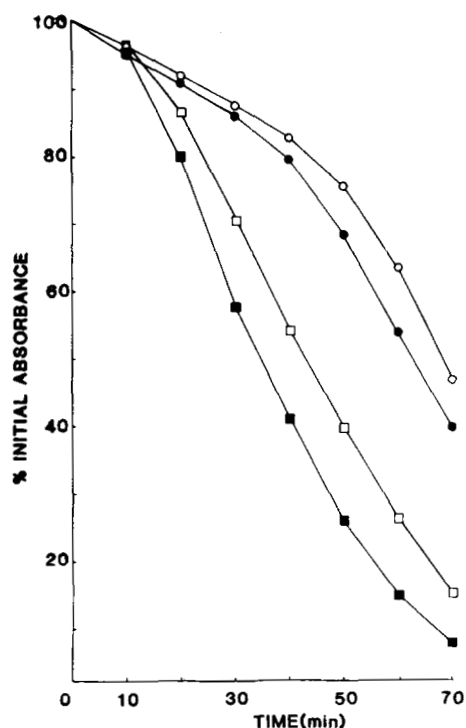


FIG. 2. Effect of proteinase and/or dodecylglycerol on whole cell autolysis. *S. faecium* cells were grown up to an OD_{675} of 0.5–0.6, harvested, and washed as described in the legend to Fig. 1. These were suspended in phosphate buffer (0.3 M, pH 6.7) and treated with trypsin (1 μ g/ml) (●), 20 μ g of dodecylglycerol/ml (□), both (■), or nothing (○) in a final volume of 3 ml and incubated at 37 °C. Autolysin activity was followed by measuring decrease in turbidity at 675 nm.

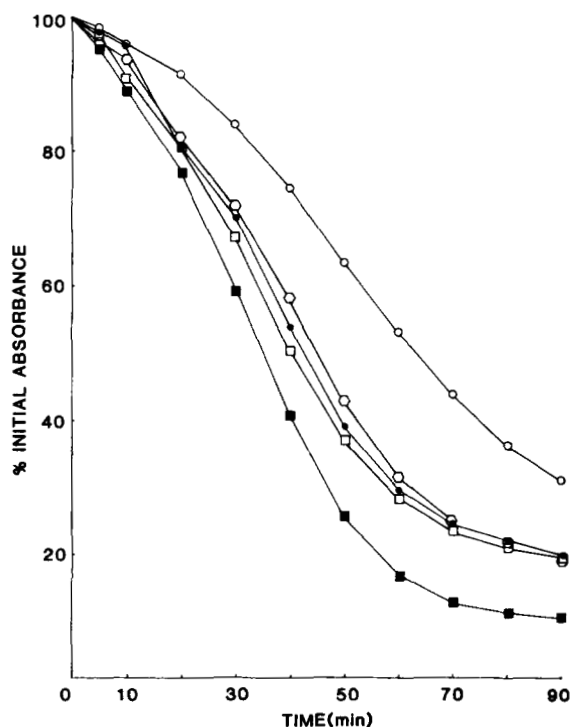


FIG. 3. Reversal of dodecylglycerol-stimulated autolysis activity by several proteinase inhibitors. *S. faecium* cells were prepared for autolysin assay as described in the legend to Fig. 1. The cells were treated with either zero (○) or 20 μ g of dodecylglycerol/ml (■). In addition, dodecylglycerol-treated cells received 100 μ g/ml of TLCK (□), TPCK (●), or antipain (△). These were incubated at 37 °C, and decrease in turbidity was followed spectrophotometrically at 675 nm.

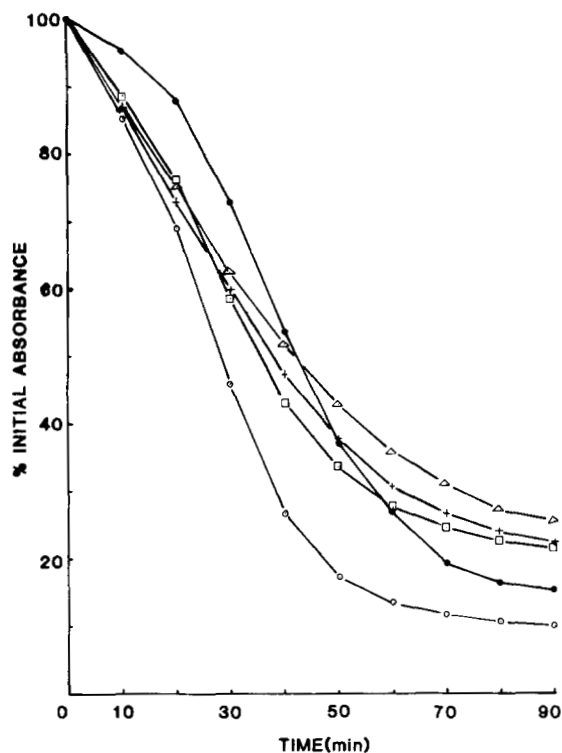


FIG. 4. Concentration-dependent reversal of dodecylglycerol-stimulated autolysis by proteinase inhibitor. *S. faecium* cells were grown, harvested, and prepared for autolysin as described in the legend to Fig. 1. Varying concentrations of TPCK were added to the buffer. ●, control; ○, dodecylglycerol (20 μ g/ml); □, dodecylglycerol (20 μ g) and TPCK (75 μ g/ml); +, dodecylglycerol (20 μ g) and TPCK (100 μ g/ml); △, dodecylglycerol (20 μ g) and TPCK (125 μ g/ml). Autolysin was measured as described in previous figure legends.

much greater extent than similar supernatants from untreated cells (Fig. 1). The autolysin activity was measured in walls from cells that had never been exposed to dodecylglycerol. Since in Fig. 1 the protein in the supernatant from treated cells was 0.50 mg and in the untreated control it was 1.1 mg, the difference of the autolysin activity between treated and untreated expressed on a per mg protein basis is actually greater than indicated in Fig. 1 (185 units untreated and 566 units dodecylglycerol-treated; a unit is that amount of enzyme that brings about a change of 0.0001 $OD^1/h/mg$ of protein).

Correlation between the Effects of Trypsin and Dodecylglycerol on Whole Cell Autolysis—Trypsin and other proteolytic enzymes are known activators of the autolysin activity of *S. faecium* (3). Treatment of whole cells of *S. faecium* with trypsin stimulates autolysin activity (Fig. 2) in a manner that mimics the stimulation of the enzyme by dodecylglycerol. The stimulation from each effector is additive (Fig. 2).

Reversal of the Stimulation by Dodecylglycerol of Autolysin Activity by Proteinase Inhibitors—The addition of a commonly known proteinase inhibitor, such as TLCK, TPCK, or antipain to the incubation assay reverses the stimulation of autolysin activity by dodecylglycerol (Fig. 3). This reversal is dependent on the concentration of the proteinase inhibitor (Fig. 4).

Stimulation of Proteinase Activity of *S. faecium* by Dodecylglycerol—The above data suggest that the proteinase activity of *S. faecium* is altered by dodecylglycerol. The direct mea-

¹ The abbreviations used are: OD, optical density; TLCK, *N*^α-tosyl-L-lysyl chloromethyl ketone; TPCK, tosylamido-2-phenylethyl chloromethyl ketone.

TABLE I
Effect of proteolytic inhibitors on dodecylglycerol-stimulated proteolysis in *S. faecium*

Inhibitors	Control	Dodecylglycerol (20 μ g/ml)
None	184.0 \pm 2.8 ^a	686.6 \pm 9.8 ^a
EDTA (10 mM)	185.9 \pm 6.8	676.0 \pm 10.3
TLCK (100 μ g)	134.6 \pm 4.6	506.0 \pm 7.1
TPCK (100 μ g)	106.4 \pm 4.7	404.0 \pm 4.1
Antipain (100 μ g)	80.7 \pm 4.1	349.0 \pm 4.9
PMSF ^b (100 μ g)	108.9 \pm 5.9	330.0 \pm 4.4

^a Net increase in absorbance at 280 nm/h/mg of protein ($\times 0.001$). The assay was performed as described under "Experimental Procedures."

^b PMSF, phenylmethylsulfonyl fluoride.

surement of proteinase activity of *S. faecium* and its considerable stimulation by dodecylglycerol is shown in Table I. This stimulation of the endogenous proteinase activity is reversed by various proteinase inhibitors. EDTA, which is an inhibitor of metalloproteinases, did not inhibit the proteinase of *S. faecium*.

DISCUSSION

Dodecylglycerol has been proposed to exert its antibacterial activity through the stimulation of the autolysin activity of the bacterial cell (1). Although this may not be the only mode of action of dodecylglycerol, it certainly must be considered a

primary possibility since the improper regulation of this crucial enzyme could indeed prevent normal bacterial replication and induce cell lysis. However, dodecylglycerol does not act directly on the autolysin of the cell wall of *S. faecium*, but rather through an intermediary of cytosolic (or possibly membrane) origin. Since the autolytic activity of *S. faecium* is known to be converted from an inactive to an active form by proteolytic action (3), it is likely that this intermediary is the proteinase activity endogenous to *S. faecium*. The data clearly show (Table I) that this proteinase can be stimulated by dodecylglycerol and that this stimulation can be reversed by specific proteinase inhibitors, which also reverse the stimulation of autolysin activity by dodecylglycerol. We therefore conclude that one primary antibacterial mode of action of dodecylglycerol is to stimulate the proteinase of *S. faecium*, which in turn activates autolysin activity and prevents bacterial growth.

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