Spontaneous Dissolution of Isolated Cell Walls from *Listeria monocytogenes* Strain A4413[1] (36851)

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A restricted number of strains from several bacterial genera yield cell walls which undergo spontaneous dissolution. This instability, in so far as it has been studied, appears due to the action of one or more degradative enzymes (autolysins) intimately associated with wall material (1).

Tinelli (2, 3) has shown that Strains NCTC 10 357 (type 1) and IP A. 106 (type 1) of *Listeria monocytogenes* yield labile wall preparations. To our knowledge, this is the only demonstration of wall instability in this genus. It is not clear whether this is a peculiarity of these strains, or whether the propensity for wall instability is shared by other members of the genus.

The present report extends these findings to *L. monocytogenes* Strain A4413, one that is of human derivation and widely used in studies of pathogenesis.

Materials and Methods. All chemicals were reagent grade. Turbidity changes of wall suspensions were recorded as optical densities at 655 nm (OD_{655}) on a Coleman Model 9 nephelocolorimeter. Strain A4413 was kindly supplied by Dr. Martin Wilder, University of Massachusetts at Amherst. It was maintained on tryptose agar (Difco) slants. Slants were used to inoculate starter cultures consisting of 25 ml brain–heart infusion broth (BHI, Baltimore Biological Laboratories). These were incubated at 35° on a water bath-shaker (100 excursions/min, 1 in. stroke).

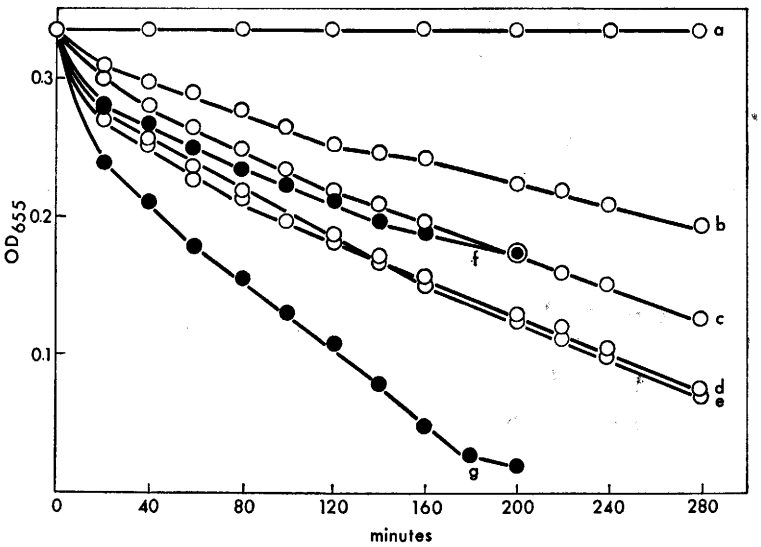

FIG. 1. Rates of wall dissolution in buffers. (O) Walls from 9 hr cells at (b) pH 5.2, (c) 6.5, (d) 7.0 and (e) 8.0; (●) walls from 4 hr cells at (f) 5.2 and (g) 8.0. The concentric point shows the end of curve (f) and continuation of curve (c). Curve (a) shows stabilization of both 4 and 9 hr walls after heat treatment.

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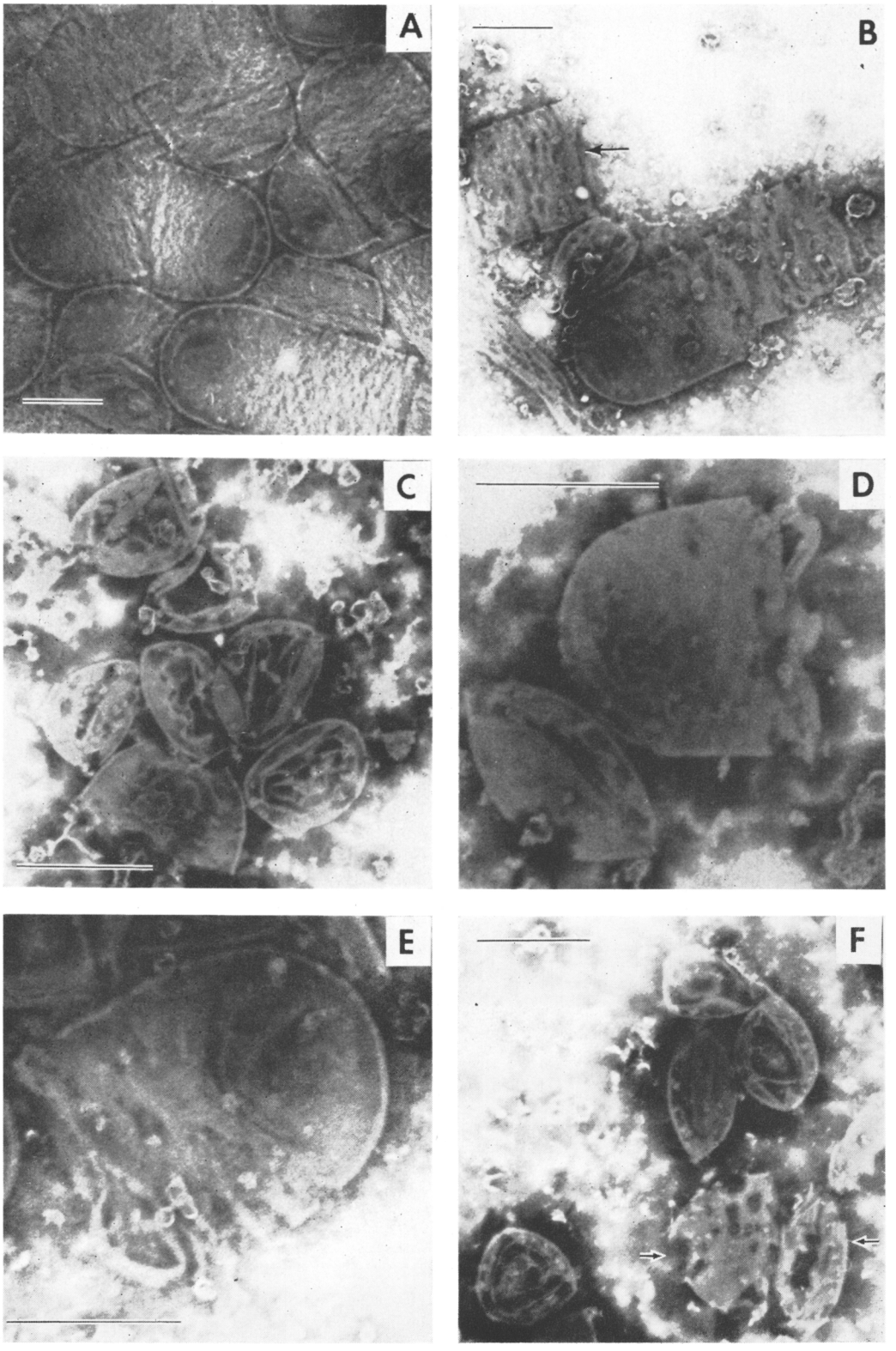
A
B
C
D
E
F

After 9 hr 2.5 ml (about 1.5×10^{10} cells) were used to inoculate each 250 ml unit of BHI; 6 such units were used to provide cells for wall preparation. Incubation was performed as for starter cultures. Under these conditions, cells routinely entered the log phase just before 4 hr and reached peak population (terminal log phase) in 9 hr.

Unless specified, subsequent steps were performed at 2 to 5°. Cultures at 9 or 4 hr postinoculation were centrifuged at 4080*g* for 15 min. Sedimented cells were washed once with succinic acid–NaOH buffer (pH 5.8, 0.05 *M*) and resuspended in buffer. This suspension was standardized to give an OD_{655} of 1.0 at 1:100 dilution. Undiluted standardized suspension was cooled to 1° and the cells disrupted with a Biosonik III ultrasonic system, using the macroprobe at full intensity. Cells from 9 and 4 hr cultures required 15 and 7 min, respectively, to reach 90% disruption, as measured by OD_{655} loss and phase-contrast microscopy. The maximum temperature reached during sonication was 10°. Intact cells were sedimented (1085*g*, 15 min) and the supernatant fluid recentrifuged (27,000*g*, 8 min). Residual intact cells sedimented as a central pellet surrounded by a translucent pellet of cell walls. The latter was removed, washed 4 times with 80 ml buffer volumes and twice with distilled water. Portions of the final pellet were rapidly suspended (OD_{655}, 0.34) in the following ice-cold buffers: succinic acid–NaOH (pH 5.2, 0.05 *M*); and 0.1 *M* phosphate at pH 6.5, 7.0 and 8.0, respectively. Portions of the suspensions were raised to 65° for 10 min. About 40 ml and 6 ml of total suspension were respectively obtained from 1.5 liters of 9 or 4 hr culture. Such suspensions contained essentially no intact cells as determined by phase-contrast microscopy. No discernible cytoplasmic contamination was seen in the electron microscope.

Wall suspensions in duplicate or triplicate were rapidly raised to 35° and loss of optical density followed over a 280 min period at this temperature. No growth of *Listeria* or contaminants was observed in this short time. Unheated suspensions were sequentially sampled for electron microscopy.

Grids (300 mesh, coated with parlodion and carbon) received a drop of sample. After 60 sec, excess fluid was removed and replaced with a drop of 1% phosphotungstic acid, which was removed after 10 sec. Grids were immediately examined in a Phillips Model 200 electron microscope. The procedure gave a combination of positive and negative staining which revealed considerable topographical detail.

Results. In buffer suspension, cell walls of Strain A4413 proved unstable, as evidenced by progressive loss of turbidity. Walls from terminal log phase cells lost 80% of initial optical density at pH 7 and 8 in the 280 min period; at pH 5.2, 40% was lost. Walls from early log phase cells also lost density maximally at pH 8 and minimally at 5.2. However, overall rate at both these pH levels was strikingly faster; 90 and 50%, respectively, were lost in 200 min or less. Distinct rate changes were observed, particularly during the slower dissolution of walls from terminal log phase cells. Heated cell walls did not lose turbidity. Replicate samples showed variation of less than 0.01 OD unit at any given point, and curves were entirely reproducible in 10 separate experiments. The results of 1 such experiment are shown in Fig. 1.

Morphological changes during dissolution were similar for both types of walls. Significant structural deterioration was present even immediately after preparation, involving lateral stratification of a superficial layer, with stripping of this layer beginning at the

FIG. 2. Morphology of degradation: (A) shows lateral stratification and initial stripping at poles, already present in freshly prepared walls. (B) shows preferential degradation and breaking off (arrow) of the main wall body from the polar cap, resulting in (C) stripped caps. (D,E) show stages in the destruction of polar caps; note unwinding at edges distal to the pole. (F), terminal destruction of polar caps (arrows). All line markers represent 0.5 μ. The sequence shown was common to both 4 and 9 hr walls.

polar caps. A residual skeleton structure was thus revealed. As incubation progressed, the stripping process extended to the main body of the wall, and appeared to involve "unwinding" of stratified layer. Polar caps resisted degradation more than the main wall body; stripped polar caps predominated late in the process. They were not destroyed until the end of the observation period. These results are shown in Fig. 2.

Discussion. Although Strain A4413 has been extensively studied, the lability of walls from cells of this organism appears to have escaped notice. Dissolution was pH dependent with the optimum between pH 7 and 8, and was precluded by mild heat treatment. Together with the small but reproducible rate changes observed, these findings suggest the sequential action of several degradative enzymes acting on multiple sites. A similar conclusion has been reached by Tinelli (3) on the basis of chemical analysis of solubilized end-products. The difference in rate between early and peak log phase walls may indicate that the former gave a larger ratio of wall-associated degradative enzyme(s) to available wall substrate. This is in agreement with a suggestion made by Rogers (4) in a study of the response of staphylococci to penicillin, based on the recognition of degradative enzymes as essential to the insertion of new units into the growing wall.

The morphological basis of degradation also appears related to successive destruction of multiple layers. The *Listeria* wall is quite complex, containing teichoic acid (3) and possibly lipopolysaccharide (5) in addition to basal peptidoglycan. However, the relation of our morphological findings to these components is uncertain at present. The slower degradation of polar caps suggests differences in structure or enzyme content in this portion of the wall compared with the main body. A similar finding has been made with a strain of *B. subtilis* (6).

Summary. Cell walls from *Listeria monocytogenes* Strain A4413 underwent spontaneous dissolution between pH 8 and 5.2; the optimum was between pH 7 and 8. Walls from early log phase cells were degraded more rapidly than walls from terminal log phase cells. Dissolution could be precluded by mild heat treatment, and appeared related to the destruction of multiple layers of wall structure.

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