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Note

Turnover of cell wall in *Listeria monocytogenes*

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Listeria monocytogenes is a Gram-positive, rod-shaped bacterium that frequently exhibits pathogenicity. Individuals who are immunosuppressed tend to be more prone to *Listeria* infections (reviewed by Nieman and Lorber[1]). The cell surface of *L. monocytogenes* appears to be distinct, differing considerably from that expected of a Gram-positive bacterium. Components characteristic of lipopolysaccharide[2,3] have been isolated from the cell wall of *Listeria*. In addition, materials possessing endotoxin-like properties have been isolated from phenol–water extracts of the bacterium[4].

Cell-wall components of *L. monocytogenes* elicit several kinds of biological response. Campbell *et al.*[5,6] showed that the wall fraction possesses mitogenic activity in murine lymphocytes, and similar results were described by Ivanyi[7]. Baker *et al.*[8] found that purified cell-walls of *L. monocytogenes* can activate complement, and can induce chemotaxis of human, polymorphonuclear leukocytes. Cell-wall fraction also appears[9] to decrease the resistance of mice against subsequent challenge by live *L. monocytogenes*. Koch *et al.*[10,11] recently predicted that the cell walls of all Gram-positive bacilli should turn over; this prediction, and the possibility that cell-wall turnover-products could mediate some of the reported biological reactions, stimulated experiments to detect turnover in *L. monocytogenes*.

EXPERIMENTAL

L. monocytogenes CAP-B1, a hospital reference-strain, was obtained from Dr. J. Snyder, Norton-Children's Hospital, Louisville. The cells were maintained on human-blood–agar slants. For turnover studies and for incorporation experiments, the bacteria were grown in antibiotic medium 3 (Penassay broth), Difco Laboratories, Detroit, MI. The cells were aerated by shaking in a rotary incubator at 37°.

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Exponentially growing cells (1400 mL) were pulsed with 1.0 μCi/mL (260 mCi/mmol; final activity) of 2-acetamido-2-deoxy-D-[1-^{3}H]glucose (GlcNAc) for two generations. The cells were then recovered by centrifugation, washed three times with distilled water, and freeze-dried. The cells (10.0 mg, 40,197 c.p.m./mg) were subjected to a modified, Park–Hancock[12–14] fractionation scheme. The walls were suspended in 5.0 mL of 3% (wt/vol) sodium dodecyl sulfate (SDS), and heated for 20 min at 100°. The cells were centrifuged, and washed several times with distilled water. All supernatant liquors were combined. The residue was subjected to extraction with 5 mL of 5% (wt/vol) trichloroacetic acid (TCA) for 18 h at 4°. The insoluble material was washed twice with 50mM sodium phosphate (pH 7.2). Supernatant liquors were combined for scintillation counting. The residue was then incubated with 5 mL of pronase (50 μg/mL) for 4 h at 37°. Insoluble materials were again washed twice with the phosphate buffer, and the residue was then incubated with egg-white lysozyme (50 μg/mL) for 18 h at 37°. Finally, the insoluble material remaining after the lysozyme treatment was washed with buffer, and digested with mutanolysin[15] (M-1 enzyme, 50 μg/mL) for 4 h at 37°. We have been unable to detect proteolytic activity in our mutanolysin preparation by using [^{14}C]-labeled hemoglobin as the substrate.

Cell-wall turnover experiments were conducted according to previously described procedures[13,16]. Cells were labeled with radioactive GlcNAc, washed with warm medium, and suspended in fresh growth-medium. Samples were removed at intervals, filtered on 0.45-μm Millipore filters, washed with distilled water, and dissolved in a nonaqueous solvent containing a scintillant. In some experiments, non-radioactive GlcNAc was added as a "chase", following the incorporation of radioactive GlcNAc.

Electron microscopy was performed on cell walls stained with 1% (wt./vol.) uranyl acetate. Procedures for the preparation of samples have been described by Fan *et al.*[17]. The specimens were examined in a Siemens Elmiskop I electron microscope.

RESULTS AND DISCUSSION

The turnover of cell wall in a bacterium can be measured, provided that a convenient marker is available. Some organisms contain diaminoheptanedioic (diaminopimelic) acid in their cell walls, and the amino acid can be used as a relatively specific probe for wall-metabolism studies. Several workers have described the use of GlcNAc in assessing the turnover of peptidoglycan in bacteria. Exponentially growing cultures of *L. monocytogenes* were pulsed for approximately two generations with [^{3}H]GlcNAc. The cells were washed, freeze-dried, and subjected to a modified, Park–Hancock, fractionation scheme, in order to determine the subcellular distribution of radioactivity. The results (see Table I) show that most of the label was in the fraction resistant to solubilization by SDS, TCA, and pronase. Lysozyme, which solubilizes the peptidoglycans of many bacteria, was not highly effective in releasing

TABLE I

DISTRIBUTION OF RADIOACTIVE 2-ACETAMIDO-2-DEOXY-D-GLUCOSE IN CELL FRACTIONS OF *Listeria monocytogenes*

Treatment	*Amount solubilized*[a]
Sodium dodecyl sulfate	10,633 (2.6)
Trichloroacetic acid	9,675 (2.4)
Pronase	3,450 (0.8)
Lysozyme	17,075 (4.1)
Mutanolysin	371,555 (90)

[a]The numbers in parentheses indicate the per cent of the total radioactivity recovered. The amount of radioactivity recovered was 412,388 c.p.m., or 102.6% of the amount of radioactivity added. Results similar to these were obtained when different amounts of cells were subjected to the fractionation procedures.

the label from the walls. In contrast, mutanolysin, a glucosaminidase from *Streptomyces globisporus*[15], quantitatively released the label from the wall residues. These findings support the results of an earlier study in which it was shown[14] that mutanolysin could solubilize the lysozyme-resistant, cell walls of *Streptococcus mutans*. As regards the sensitivity of the cell walls of *L. monocytogenes* to lysozyme, Ghosh and Murray[18] found that the walls of six of eight strains could be solubilized by the enzyme. Pancreatic lipase rendered the resistant walls susceptible to lysozyme through some as-yet-unknown means.

Fig. 1. Electron micrograph of *Listeria monocytogenes* cell walls. (The walls had been extracted with SDS and TCA, and then digested with pronase; magnification of 12,375.)

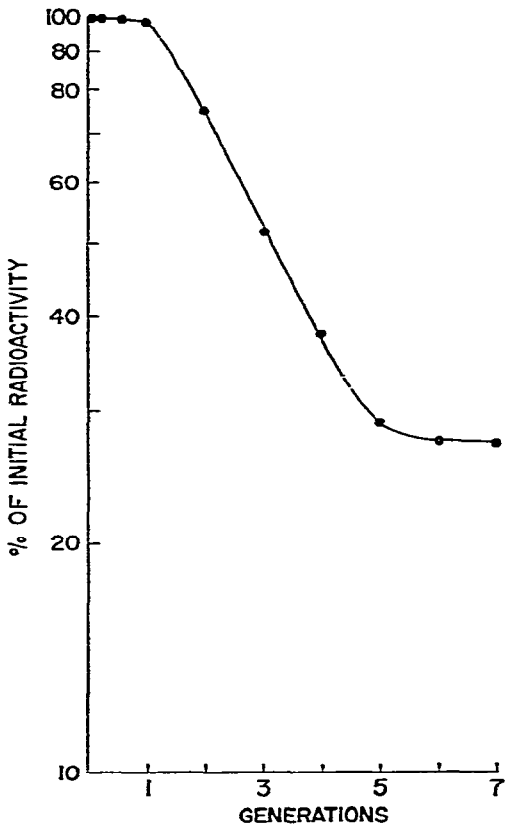

Fig. 2. The exponential turnover of cell walls of *L. monocytogenes*. {Cells were pulsed for 0.5 generation with 1 μCi (final activity) of [^{3}H]GlcNAc/mL. The cells were rapidly filtered, washed with prewarmed growth-medium, and finally suspended in fresh growth-medium. Samples were removed at intervals, filtered, and washed, and the radioactivity was determined by scintillation counting. The initial sample contained 9,400 c.p.m.}

In order to confirm that the residues of thoroughly extracted and enzymically hydrolyzed samples were actually cell walls, intact bacteria were disrupted[13] and treated according to the modified Park–Hancock fractionation procedures (see Table I). Residues were subjected to electron-microscope observations. In Fig. 1 is shown an electron micrograph of a specimen, revealing the characteristic outlines of cell walls of bacilli.

The composite results of the foregoing experiments show that GlcNAc is a highly specific label for cell wall of *L. monocytogenes*. The results suggest that cells labeled with [^{3}H]GlcNAc may be useful in establishing whether the walls of *L. monocytogenes* can turn over during growth. In Fig. 2, results are presented which show that approximately 25% of the label is lost per generation from a [^{3}H]GlcNAc-labeled population of bacteria. This rate of "turnover" is less than that for *Bacillus subtilis*[13,16], but approximately the same as that for *Lactobacillus acidophilus*[19]. Two

features of turnover of walls in *L. monocytogenes* appear to be characteristic for Gram-positive rods. The exponential loss of label from the cells follows a lag of approximately one generation after the removal of the nonincorporated [^{3}H]GlcNAc (see Fig. 2). It is probable that, in *L. monocytogenes*, as in *B. subtilis*, cell wall migrates from the inner to the outer face during growth[20,21]. The wall on the outer face then becomes susceptible to the actions of autolysins[16]. Secondly, turnover tends to slow markedly after ~3–4 generations, leaving a large amount of label cell-associated. We have also observed that, in *B. subtilis*, ~5–10% of the wall does not turn over, even after many generations[13]. We presented evidence to show that the turnover-resistant, wall material was found at cell poles and not on cell cylinders[13]. Recent studies have predicted, on theoretical grounds, that exponential turnover of walls is more rapid in cell cylinders[10,11]. Tyrrell *et al.*[22] noted that the poles of cell-wall preparations of *L. monocytogenes* tend to retard autolysis. The results cannot be used to establish whether the endotoxin-like components turn over. In Gram-negative bacilli, there is no evidence for turnover of lipopolysaccharide during cell division.

L. monocytogenes possesses an active, autolytic system[22,23]. The organism probably sheds wall when it is phagocytized. The intracellular presence of the products of autolysin(s) may influence the subsequent behavior of cells involved in immune responses.

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