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Note

Incorporation of 2-acetamido-2-deoxy-D-glucose into the peptidoglycan of *Streptococcus mutans*

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In order to study and characterize specific, cellular constituents for structure and function, it is expedient to use specific markers for the constituents. This is particularly important when examining the role of bacterial, macromolecular components in pathogenesis. For example, the adherence of *Streptococcus mutans* to solid surfaces can conveniently be monitored by use of intact cells grown in a medium[1] enriched in tritiated thymidine. Because the DNA does not leak from the cells, it is possible to assess accurately the extent of adherence of the labeled bacteria to smooth surfaces. When considering precisely which cellular constituents are responsible for interaction with solid surfaces, it is much more difficult to obtain reliable information, and it may be necessary to employ purified fractions before interpretations can be made. In previous work, we suggested[1,2] that the cell wall is, or peptide groups covalently bound to the peptidoglycan are, involved in the initial attachment of *S. mutans* to saliva-coated hydroxylapatite (SHA). Adherence of isolated cell-walls to SHA would have to be observed in order to substantiate these claims. To date, specific markers for cell walls of *S. mutans* are unavailable; Boothby *et al.*[3] employed radioactive amino acids as markers for peptidoglycan in *S. faecalis*. Because the cell wall of *S. mutans* contains tightly or covalently bound polypeptides and other macromolecules having unusual biological and immunological characteristics[1,2,4], the use of amino acids as cell-wall labels must be more carefully examined before the validity of the method can be established.

Extensive use has been made of 2-acetamido-2-deoxy-D-glucose (GlcNAc) as a precursor for cell-wall peptidoglycan in some non-streptococcal bacteria[5], but its use in streptococci has not been adequately evaluated. Dezélée and Shockman, however, reported[6] that neither 2-amino-2-deoxy-D-glucose nor GlcNAc was readily incorporated into the cell wall of *S. faecalis*. We now propose the use of [^{14}C]GlcNAc

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as a specific, labeled precursor for the cell wall of *S. mutans*, and we have demonstrated that some of the problems associated with the fractionation of *S. mutans* can be overcome.

EXPERIMENTAL

Cells[2] were grown in brain-heart infusion-broth (Difco, Detroit, MI) containing 0.05 μCi/mL of [^{14}C]GlcNAc (40 μCi/mg). The cells were harvested, washed four times in cold water, and freeze-dried. The labeled cells (10–20 mg samples, dry weight) were suspended in the extractant (5.0 mL). After incubation, recoveries were made by centrifugation, and the residues were subjected to the next extraction, or hydrolysis procedure. Trypsin digestion was conducted in 75mM 2-amino-2-(hydroxymethyl)-1,3-propanediol ["tris(hydroxymethyl)aminomethane"; Tris] HCl (pH 7.9) containing 1mM Ca^{2+}. The concentration of protein was 500 μg/mL. Mutanolysin ("Ml" enzyme at 100 μg/mL) was added to trypsin-insoluble material. The buffer was 0.01M Tris-malate (pH 7.1). The amount of radioactivity in the freeze-dried cells was 15,810 c.p.m./mg. Peptidase activity was not detected in the mutanolysin preparation. Assays for peptidase activity were based on the solubilization of chromophore from hide-powder azure (Calbiochem, LaJolla, CA), the conditions for the peptidase assays being 30 min at 37°, and 100 μg of soluble enzyme/mL, in 0.1M Tris-malate (pH 7.1). The labeled precursor (2-acetamido-2-deoxy-D-[1-^{14}C]glucose) was purchased from New England Nuclear, Boston, MA. The results shown are the average of four separate experiments.

Cell walls were subjected to extraction with detergents and denaturants[2], to remove possible contaminants. The walls (1.0 mg) were hydrolyzed in 0.5M HCl in a sealed, glass vial by heating for 12 h at 100°. Standards (GlcNAc and *N*-acetylmuramic acid) were similarly hydrolyzed. The hydrolyzates were dried with a stream of nitrogen, and brought to the original volume with distilled water. Samples (5 μL) were spotted on Whatman No. 1 paper, and subjected to ascending chromatography, the developing solvent being 5:1:2 (v/v/v) 1-butanol–glacial acetic acid–water.

RESULTS AND DISCUSSION

The results from a labeling experiment are described in Table I. Cells were grown in a rich medium containing [^{14}C]GlcNAc. The cells were harvested by centrifugation, washed several times with cold water, and freeze-dried. The freeze-dried, labeled cells were subjected to a modified Park–Hancock[7] fractionation scheme, in order to determine which cellular constituents contained the radioactivity. The data clearly show that [^{14}C]GlcNAc is selectively incorporated into the peptidoglycan fraction. The small amounts of radioactivity extracted into water, chloroform–methanol, and trichloroacetic acid (TCA) may represent improperly sedimented cells, or, possibly, cell-wall precursors. The absence of significant amounts of trypsin-soluble radioactivity probably reflects the general absence of glycoproteins in *S.*

TABLE I

INCORPORATION OF [^{14}C]GlcNAc INTO THE CELL WALLS OF *S. mutans* 6715

Extraction with	*Time and temperature*	*Soluble radioactivity (% of total)*	*Component solubilized*[5,7]
Water	30 min at 22°	0.3	water-soluble precursors
$CHCl_3$–MeOH (2:1, v/v)	60 min at 22°	0.7	lipids, nonpolar compounds
Trichloroacetic acid (TCA, 5%)	4 h at 3°	0.5	amphipathic molecules, RNA, polysaccharides
TCA (5%)	1 h at 60°	0.6	nucleic acids, small polypeptides
75% (v/v) EtOH	10 min at 22°	0.1	TCA-degradation products
Trypsin	18 h at 37°	1.5	protein
Mutanolysin	14 h at 37°	96.3	cell wall

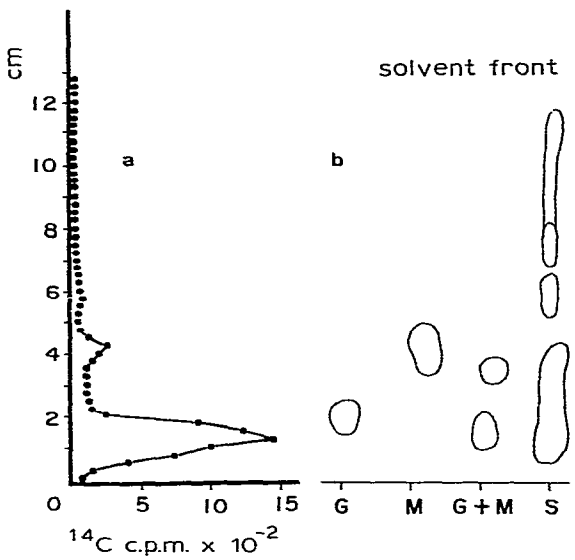

Fig. 1. Chromatography of a [^{14}C]GlcNAc-labeled, cell-wall hydrolyzate. Cell walls were subjected to extraction with detergents and denaturants[9], to remove possible contaminants. The walls (1.0 mg) were hydrolyzed in 0.5M HCl in a sealed, glass vial by heating for 12 h at 100°. Standards (GlcNAc and *N*-acetylmuramic acid) were similarly hydrolyzed. The hydrolyzates were dried in a stream of nitrogen, and brought to the original volume with distilled water. Samples (5 μL) were spotted on Whatman No. 1 paper, and subjected to ascending chromatography. The developing solvent was 5:1:2 (v/v/v) 1-butanol–glacial acetic acid–water. Key: a, profile of radioactivity on the chromatogram following development; b, visibilization of cell-wall digest and controls with ninhydrin (G = 2-amino-2-deoxy-D-glucose, M = muramic acid, and S = cell-wall sample).

mutans, and the non-conversion of GlcNAc into cellular protein. It should be noted that *S. mutans* is actually a "group" of bacteria having similar, phenotypic characteristics, and it is therefore possible that results obtained with *S. mutans* 6715 may not be extrapolatable to all *S. mutans* strains. *S. mutans* strain 6715 has been used extensively as a "standard" for many different kinds of studies.

In order to confirm that the labeled GlcNAc was incorporated into peptidoglycan, cell-wall samples were hydrolyzed, and the hydrolyzate was subjected to paper chromatography. The results (see Fig. 1) reveal that a major and a minor peak of radioactivity were present, corresponding to 2-amino-2-deoxy-D-glucose and muramic acid. A broad band of ninhydrin-positive material (assumed to be amino acids) migrated ahead of the muramic acid. The results showed that GlcNAc is specifically incorporated into peptidoglycan components.

One difficulty with the use of labeled, metabolic precursors for markers of cell walls in streptococci is the resistance of the walls to such bacteriolytic enzymes as egg-white lysozyme[8-10]. Because all of the wall material cannot be enzymically solubilized, it has heretofore been impossible to evaluate fractionation schemes. The use of mutanolysin, a muramidase-like enzyme, to effect the solubilization of cell walls has made possible the devising of procedures for assessing the fate of an incorporated, macromolecular precursor in *S. mutans*. The enzyme, isolated from *Streptomyces globisporus*[11,12], was shown to be devoid of peptidase activities. Because the enzyme has the capacity to solubilize cell-walls from unrelated species, it should prove useful in cell-wall structure–function studies on many bacteria which produce peptidoglycans that are refractory to bacteriolytic enzymes. Cell walls of *S. mutans* specifically labeled with GlcNAc should be valuable to investigations concerning peptidoglycan biosynthesis and turnover, for determining the effects of antibiotics on cell growth, and for characterizing the mechanism of the adherence of the bacterium to smooth surfaces.

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