SHORT COMMUNICATION

The Occurrence of Trehalose in *Micrococcus* Species

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Five strains of *Micrococcus*, representing three species, accumulated trehalose when grown in the presence of glucose. Trehalose was not found in two *Staphylococcus* species or in *Planococcus citreus*.

INTRODUCTION

α,α -Trehalose (α -D-glucopyranosyl α -D-glucopyranoside) is a non-reducing disaccharide found widely in insects, invertebrates, algae, fungi and yeasts, where its function is probably that of a reserve material (Elbein, 1974). Trehalose is also found in the so-called 'higher' bacteria, e.g. mycobacteria and corynebacteria, but in these organisms most of it occurs as esters with long-chain fatty acids and in this form it acts as a structural component (Elbein, 1974). Stjernholm (1958) found that propionibacteria converted glucose into trehalose, and Fischer & Kriegelstein (1967) isolated a derivative of an isomer of α,α -trehalose, namely, 3-O- α -D-glucopyranosyl- α,β -trehalose, from *Streptococcus faecalis*. In this communication we report the occurrence of α,α -trehalose in certain species of *Micrococcaceae* grown in the presence of glucose.

METHODS

Organisms. All bacteria apart from *Planococcus citreus* were obtained from the National Collection of Type Cultures, London; *P. citreus* was obtained from the National Collection of Marine Bacteria, Aberdeen.

Growth of bacteria. Most of this work was done with *Micrococcus luteus* NCTC 2665 (formerly *Micrococcus lysodeikticus*). This bacterium was grown in Oxoid nutrient broth (code CM1) (10 g l⁻¹) in 0.1 M-sodium phosphate buffer, pH 7.1. All other organisms were grown in Oxoid nutrient broth (10 g l⁻¹) in water. The medium for *P. citreus* was supplemented with NaCl (2.5 g l⁻¹). Glucose was added to media as described in the Results.

Organisms were grown at 30 °C in baffled conical flasks rotated at 120 rev. min⁻¹. Bacteria were harvested in a Sorvall RC-2B ultracentrifuge at 0 to 2 °C and washed twice with ice-cold water.

Bacterial density. Bacterial densities were measured by drying known volumes of aqueous suspensions to constant weight.

Carbohydrate determination. Total hexose in cells and in extracts of cells were determined as glucose by the anthrone method of Hodge & Hofreiter (1962). Reducing sugar was determined by the method of Somogyi as modified by Nelson (Dische, 1962). Free glucose in solution was determined using a Boehringer blood-sugar ('GOD-Perid') test kit. Glucose in chemical combination was released by hydrolysis with 1 M-H₂SO₄ for 4 h at 100 °C and estimated using a test kit (as above) after neutralization of the hydrolysate by addition of concentrated K₂HPO₄ solution.

Ion-exchange chromatography. Extracts of cells were deionized by passing them through columns of the mixed-bed exchanger Amberlite MB-3.

Gel chromatography. This was performed with Sephadex G-10 (Pharmacia) packed in a glass column to give a bed, 55 cm × 2 cm diam. The Sephadex was equilibrated with 10 mM-sodium phosphate buffer, pH 7.0, before loading the material to be chromatographed (dissolved in the same buffer). Material was

eluted from the column at 6 ml h⁻¹ using the same buffer, and 5 ml fractions were collected. Samples of each fraction were treated by the anthrone method to detect hexose-containing material. Dextran (molecular weight 9400), maltotriose and glucose were used to calibrate the column.

Paper chromatography. One-dimensional descending paper chromatography was performed with Whatman no. 1 paper using ethyl acetate/pyridine/water (13:5:4, by vol.) as solvent. Compounds on the chromatogram were detected by staining with silver nitrate solution (Trevelyan *et al.*, 1950).

Proton magnetic resonance spectra. Mr G. C. Brophy, School of Chemistry, University of Sydney, obtained proton magnetic resonance spectra using a Varian XL-100 spectrophotometer.

Attempted isolation of glycogen. Two methods were used in an attempt to show the presence of glycogen in *M. luteus* grown in the presence of glucose. One was based on an extraction of cells with a hot, concentrated solution of KOH followed by precipitation of glycogen from the extract with 70 % (v/v) ethanol (Segel *et al.*, 1965). The other was based on a method applied successfully to many types of cell and involved the centrifugation at 100000 g for 1 h of a soluble extract of mechanically disrupted cells; glycogen sediments under these conditions (Yamaguchi *et al.*, 1974).

Isolation of hexose-containing material accumulated when *M. luteus* was grown in the presence of glucose. Washed bacteria which had been grown in the presence of 1 % (w/v) glucose were suspended in water (20 mg dry wt ml⁻¹) and the suspension was heated at 100 °C for 5 min. The suspension was cooled, centrifuged to remove insoluble material and the extract was filtered through a Millipore filter (0.22 µm diam. pores). The filtrate was lyophilized and the dried filtrate (hexose content, 37 %) was dissolved in distilled water (170 mg in 4 ml, representing the yield from 1 l of culture harvested late in the growth phase). The solution was passed through a column of Amberlite MB-3 (23 cm × 0.9 cm diam.) and the column was eluted with water at 10 ml h⁻¹; 82 % of the hexose-containing material in solution passed through the column without retention. The eluate containing this material was lyophilized. The dried eluate (hexose content, 102 %) was dissolved in 1 ml 10 mM-sodium phosphate buffer, pH 7.0, and the solution was loaded on to a column of Sephadex G-10. Hexose-containing material was eluted from the column in a single, sharp peak emerging between the points at which maltotriose and glucose were eluted in a separate experiment. The fractions containing hexose were combined and deionized with Amberlite MB-3 and the solution was lyophilized. The hexose content of the dried material was 102 % and the hexose:glucose ratio was 1:1. The reducing value was 0.015 of that given by a similar mass of glucose. Paper chromatography of the material gave a single, major spot with identical mobility to authentic trehalose and a trace of glucose which accounted for the slight reducing properties of the material. Partial acid hydrolysis of the material (1 M-H₂SO₄, 100 °C, various times up to 4 h) yielded no compounds other than glucose as detected by paper chromatography.

The material was crystallized from 80 % (v/v) ethanol; its melting point was 97 °C. Authentic trehalose melted at 96 to 97 °C. The proton magnetic resonance spectrum of the crystalline material in deuterium oxide was identical with that of authentic trehalose.

RESULTS

Hexose content of Micrococcus luteus NCTC 2665

The hexose content of *M. luteus* NCTC 2665 grown in nutrient broth was consistently 5 to 6 % of the dry weight of whole cells with little variation over the growth and stationary phases. The hexose content increased to 12 to 13 % of the dry weight when the medium contained 1 % (w/v) glucose.

No evidence for the presence of glycogen in *M. luteus* grown in the presence of 1 % (w/v) glucose could be obtained. Addition of ethanol to an extract of alkali-treated cells produced a precipitate containing little hexose; most hexose in the extract remained soluble and diffused through a dialysis sac. Likewise, centrifugation of soluble extracts from cells disrupted in a Mickle disintegrator produced a sediment containing little hexose; most hexose in the extract remained soluble and was diffusible.

Washed cells of *M. luteus* were suspended in distilled water and the suspension was heated at 100 °C for 5 min. The cells were removed by centrifugation. The extracts contained only a trace of hexose-containing material if the cells had been grown in nutrient broth alone but if the cells had been grown in the presence of glucose (1 %, w/v), 50 to 60 % of cellular hexose was extracted by the hot water. The percentage of cellular hexose extracted was independent of the concentration of cells in suspension over the range tested, i.e. 4 to 30 mg dry wt (ml suspension)⁻¹. Extraction of hexose was complete after heating for 2 min

but heating was continued for a further 3 min to ensure that inactivation of trehalase occurred.

The hexose-containing material in the extract was purified by chromatography and crystallized, as described in Methods. The compound was found to be of low molecular weight and its melting point, chromatographic behaviour and proton magnetic resonance spectrum showed it to be α,α -trehalose.

Hexose content of other Micrococcaceae

The organisms listed below had uniformly low hexose content (5 to 10 % for organisms 1 to 4; less than 1 % for organisms 5 to 7) when grown in nutrient broth. When the medium was supplemented with 0.5 % (w/v) glucose the hexose contents of all organisms increased, in particular those of organisms 1 to 4.

1. <i>Micrococcus luteus</i> NCTC 196	5. <i>Staphylococcus aureus</i> NCTC 8532
2. <i>Micrococcus varians</i> NCTC 7564	6. <i>Staphylococcus saprophyticus</i> NCTC 7292
3. <i>Micrococcus roseus</i> NCTC 7520	7. <i>Planococcus citreus</i> NCMB 1493
4. <i>Micrococcus roseus</i> NCTC 7523	

All organisms were allowed to grow in the presence of 0.5 % (w/v) glucose and were then harvested and washed. The washed cells were extracted with hot water and the extracts were examined by paper chromatography. Trehalose was found in copious amounts in organisms 1 to 4 and accounted for most of the extra hexose found in cells grown in the presence of glucose. Organisms 5 to 7 did not contain trehalose.

DISCUSSION

Micrococcus luteus possesses two structural components containing hexose – a glucose-rich polysaccharide found in the cell wall (Perkins, 1963) and a mannlipid found in the plasma membrane (Scher & Lennarz, 1969). When the organism is grown in the presence of glucose the hexose content of the cells approximately doubles. This increase is not due to the formation of glycogen. Growth of the organism in media containing glucose does not alter appreciably the amount of hexose deposited in the structural components mentioned above (unpublished observations). Thus, most of the increase in the hexose content of glucose-grown cells is attributable to the formation of trehalose.

Although trehalose is widely distributed in nature, where it serves primarily as a storage material, its occurrence in bacteria is considered rare (Elbein, 1974). It has been found in the free state in three species of propionibacteria (Stjernholm, 1958) and also in small amounts in the free state in mycobacteria and corynebacteria, but in these bacteria most of the trehalose is in the form of glycolipids (Elbein, 1974).

The discovery of trehalose in *M. luteus* widens the range of bacteria in which the disaccharide is found and we have examined seven other species of the family *Micrococcaceae* for their ability to form trehalose when grown in the presence of glucose. The organisms studied were selected according to the classification scheme for *Micrococcaceae* used in the eighth edition of *Bergey's Manual* (Baird-Parker, 1974). In this classification there are three genera in the family *Micrococcaceae*. Five species in the first genus (*Micrococcus*) all synthesized trehalose. Two species in the second genus (*Staphylococcus*) and one in the third (*Planococcus*) did not produce trehalose. Thus, there is a clear phylogenetic pattern in the occurrence of trehalose in *Micrococcaceae*.

The discovery of trehalose in micrococci recalls the work of Binnie *et al.* (1960) who reported that a laboratory strain of *Sarcina lutea* (i.e. *Micrococcus luteus*) accumulated an oligosaccharide containing less than 10 glucose units when grown in the presence of 2 %

(w/v) glucose. In the light of the work reported here, the compound detected by Binnie *et al.* (1960) was possibly trehalose.

The occurrence of trehalose in micrococci raises the question of the function of this compound. The most obvious function is that it serves as a storage material, but Burleigh & Dawes (1967) established that the survival of *Sarcina lutea* is not enhanced by the presence of the compound which we believe now to be trehalose. Since trehalose has a low molecular weight it must have a pronounced effect on the osmotic properties of the cell and an alternative function for trehalose is that it serves as an osmo-regulator being formed in response to the lowering of the activity of water caused by the addition of glucose to the medium. We have conducted experiments to investigate these possibilities and the results will be published later.

REFERENCES

- BAIRD-PARKER, A. C. (1974). Micrococcaceae. In *Bergey's Manual of Determinative Bacteriology*, 8th edn, pp. 478-490. Edited by R. E. Buchanan & N. E. Gibbons. Baltimore: Williams & Wilkins.
- BINNIE, B., DAWES, E. A. & HOLMS, W. H. (1960). Metabolism of *Sarcina lutea*. *Biochimica et biophysica acta* **40**, 237-251.
- BURLEIGH, I. G. & DAWES, E. A. (1967). Studies on the endogenous metabolism and senescence of starved *Sarcina lutea*. *Biochemical Journal* **102**, 236-250.
- DISCHE, Z. (1962). Color reactions based on the reducing properties of sugars. *Methods in Carbohydrate Chemistry* **1**, 512-514.
- ELBEIN, A. D. (1974). The metabolism of α,α -trehalose. *Advances in Carbohydrate Chemistry and Biochemistry* **30**, 227-256.
- FISCHER, W. & KRIEGLSTEIN, J. (1967). 3-O- α -D-Glucopyranosyl- α,β -trehalose aus *Streptococcus faecalis* III. *Zeitschrift für physiologische Chemie* **348**, 1252-1255.
- HODGE, J. E. & HOFREITER, B. T. (1962). Determination of reducing sugars and carbohydrates. *Methods in Carbohydrate Chemistry* **1**, 380-394.
- PERKINS, H. R. (1963). A polymer containing glucose and aminohexuronic acid isolated from the cell walls of *Micrococcus lysodeikticus*. *Biochemical Journal* **86**, 475-483.
- SCHER, M. & LENNARZ, W. J. (1969). Studies on the biosynthesis of mannan in *Micrococcus lysodeikticus*. *Journal of Biological Chemistry* **244**, 2777-2789.
- SEGEL, I. H., CATTANEO, J. & SIGAL, N. (1965). The regulation of glycogen synthesis in *Aerobacter aerogenes*. *Colloques internationaux du Centre National de la Recherche Scientifique* **124**, 337-355.
- STJERNHOLM, R. (1958). Formation of trehalose during dissimilation of glucose by *Propionibacterium*. *Acta chemica scandinavica* **12**, 646-649.
- TREVELYAN, W. E., PROCTER, D. P. & HARRISON, J. S. (1950). Detection of sugars on paper chromatograms. *Nature, London* **166**, 444-445.
- YAMAGUCHI, H., KANDA, Y. & IWATA, K. (1974). Macromolecular structure and morphology of native glycogen particles isolated from *Candida albicans*. *Journal of Bacteriology* **120**, 441-449.