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Release of Trehalose by Symbiotic Algae

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Abstract

A cultured strain of symbiotic *Chlorella*, NC64A, originally isolated from *Parmecium bursaria* has been discovered to translocate a non-maltose disaccharide. Based on several tests and infrared spectra, the sugar has been identified as trehalose. Translocation of the sugar *in vitro* is pH dependent. The possible role of this sugar in the biology of symbiosis is discussed.

Keywords: symbionts, symbiotic algae,*Chlorella*, trehalose

1. Introduction

The ability of certain freshwater green algae (*Chlorella* sp., Chlorophyceae) to enter into a symbiosis with some species of hydra (*Hydra viridis*) and paramecium (*Parmecium bursaria*) and a few other species of invertebrates is well-documented. The algae assume residence as endosymbionts, living and reproducing within the host's cells. Various aspects of the biology of these symbioses have been reported and are reviewed in Cook (1980, 1981, 1983). A key characteristic of the symbiotic algae is their ability to translocate soluble carbohydrate to the surrounding host cytoplasm. A variety of studies have shown that translocated carbohydrate enters the host's metabolic network and may augment host nutrition (see reviews cited above).

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The principal carbohydrate translocated *in vitro* by most symbiotic *Chlorella* strains so far studied has been identified as the disaccharide, maltose, though one strain of symbiotic *Chlorella* (838 from *Spongilla*) apparently translocates only glucose (Muscatine et al., 1967), and a symbiont from the ciliate *Climacostomum virens* translocates glucose, fructose, and xylose (Reisser et al., 1984).

During a routine analysis of product translocated by cells from a symbiotic alga (strain NC64A), a large quantity of a substance insoluble in cold 80% ethanol was obtained. Preliminary analysis using a standard test for reducing sugars and thin layer chromatography suggested the product was not maltose; further analyses revealed that the unknown product was trehalose. This is the first report of trehalose occurring as a translocation product of symbiotic algae and of trehalose being produced by a green alga (Chlorophyceae).

2. Materials and Methods

Source of algal symbionts: cells of the NC64A strain of symbiotic algae used here were originally isolated from *Parmecium bursaria* by Karakashian (1963). Subcultures of the strain were used in the reinfection studies of Pardy and Muscatine (1970). From 1971 to 1977 subcultures of the strain were maintained in continuous culture in Pardy's laboratory at U.C. Irvine, where the algae were intermittently used to infect aposymbiotic hydra. Library cultures of these algae were used as starting material for setting up cultures in Pardy's laboratory at Nebraska, where the algae has been in continuous culture from 1977 to the present and has been used periodically in various reinfection experiments.

Cell culture

Symbiotic algae (strain NC64A) were grown on 10% Bold's basal medium (Nichols and Bold, 1965) supplemented with 0.1% yeast extract and sodium glutamate (50 mM). Cultures were maintained under continuous light (25 μE $m^{-2}s^{-1}$, PAR) at 20°C with continuous shaking.

Preparation of translocated product

Cells were harvested from growth medium by centrifugation, washed with 10% Bold's inorganic basal medium and suspended in 20 ml of 10% Bold's basal medium (no organic supplements) adjusted to pH 3.2 or 7.4 with citrate-phosphate buffer (0.01 M). In all experiments the cell density was

adjusted to 1×10^8 cells/ml. After 1–2 hours of incubation, the medium was centrifuged to remove the cells. In some cases, the cell-free supernatant was further purified by filtering through a membrane filter (0.22 μm pore size).

Thin layer chromatography

Prior to analysis by thin layer chromatography, the cell-free supernatant layer was flash-evaporated at 60°C to 1 ml. The entire 1 ml sample was loaded onto a cation column (BioRad Ag 50W-X8) piggy-backed to an anion column (BioRad AG 1-X8) and eluted with glass distilled water. The neutral eluate was flash-evaporated at 60°C to 1 ml. The total carbohydrate content of the 1 ml concentrate was estimated using the anthrone method (Umbreit et al., 1972) and authentic trehalose as a standard.

Samples of the concentrated material were then co-chromatographed with authentic standards of glucose, sucrose, maltose and trehalose (Sigma Chemical Company). Ten microliter samples of sugar standards (50 mg/ml water) and the unknown were applied to standard Kodak chromatography sheets (no. 13181) coated with silical gel previously activated at 100°C. Ascending chromatography was performed in a mixture of acetone:water (17:3) until the solvent front was within approximately 1 cm of the top edge of the sheet. Spots were developed using a diphenylamine-aniline spray (4 g diphenylamine, 4 ml aniline, 100 ml acetone and 20 ml H_3PO_4 = variation A, or with 30 ml of H_3PO_4 = variation B) at 100°C.

Infrared spectroscopy

For analysis using infrared spectroscopy 100 μl of either sugar standards (50 mg/ml water) or unknowns were dried onto a barium fluoride window. Absorption spectra were determined using a Perkin Elmer 1750 Infrared Fourier Transform Spectrometer. Absorption data were analyzed using a Perkin Elmer 7500 Laboratory Computer.

Qualitative analysis

Samples of translocated material partially purified using column chromatography as described earlier were tested for the presence of reducing sugars using Benedict's solution (Umbreit et al., 1972). The ability of α-glucosidase to hydrolyze the unknown and maltose as determined by incubating a sample of the unknown in distilled water with 2 units of the enzyme (Sigma Type V, previously dialyzed overnight at pH 4.5) for 30 min at room temperature. After incubation, the medium was deproteinated with ethanol

to a final ethanol concentration of 50% and analyzed for the presence of glucose using high performance liquid chromatography.

3. Results

The final, concentrated eluate prepared from the incubation medium of cells incubated at pH 3.2 for 1–2 hr contained between 25–45 μg/ml of carbohydrate.

Data summarized in Table 1 indicate the unknown substance translocated *in vitro* by the symbionts was a non-reducing sugar resistant to hydrolysis by α-glucosidase (maltase). For example, at 1.25 mg/ml, maltose was completely converted to glucose by the enzyme, whereas neither trehalose nor the unknown produced glucose in the presence of the enzyme. Variation A of the diphenylamine developing reagent was ineffective in detecting authentic trehalose but readily reacted with maltose on thin layer chromatograms; variation B of the diphenylamine reagent was effective in detecting both maltose and trehalose on chromatograms and reacted with the unknown. Variation B of the diphenylamine reagent was used in the development of all subsequent thin layer chromatograms of the unknown translocation product. Rf values of trehalose and the unknown are given in Table 2. While the Rf values of glucose and sucrose were identical in the solvent system used in chromatography, the two sugars were readily distinguished on the basis of their color reaction products after development with the diphenylamine reagent.

Table 1. Comparison of unknown product with maltose and trehalose with respect to various qualitative tests

Test	Maltose	Trehalose	Unknown
Reduced by Benedict's solution (A)	+	–	–
Reacts with diphenylamine (Var. A)	+	–	–
Reacts with diphenylamine (Var. B)	+	+	+
Hydrolyzed by α-glucosidase to yield glucose	+	–	–

The distinctive spectra of trehalose, maltose, and the unknown are shown in Fig. 1. Table 3 compares the precise wave numbers of the peaks of trehalose, maltose, and the unknown as shown on Fig. 1. The unknown and trehalose have 16 corresponding peaks whereas maltose exhibited only 2 peaks in common with trehalose and the unknown (Table 3).

Table 2. Rf values of three sugars and unknown as determined by thin layer chromatography

Sugar	Rf
glucose	0.50
sucrose	0.50
maltose	0.37
trehalose	0.22
unknown	0.25

Table 3. Wave numbers of IR absorption peaks of maltose, trehalose and an unknown disaccharide, scanned over a range of 900–1700 cm^{-1}

Wave numbers		
Trehalose	Unown	Maltose
1293.2	1294.9	
1242.0	1240.7	
1181.5	1181.8	
		1147.0
1131.6	1132.0	
		1115.8
		1106.7
		1097.7
1091.7	1090.9	
		1076.6
1063.7	1066.4	1067.5
		1053.9
		1030.3
		1022.7
1011.9	1010.7	1010.3
961.6	962.3	

Translocation of trehalose by symbiotic algae is both light and pH sensitive as shown in Fig. 2. At pH 3.2 translocation was enhanced approximately 3 times in the dark and approximately 4 times in the light in comparison to translocation at pH 7.4

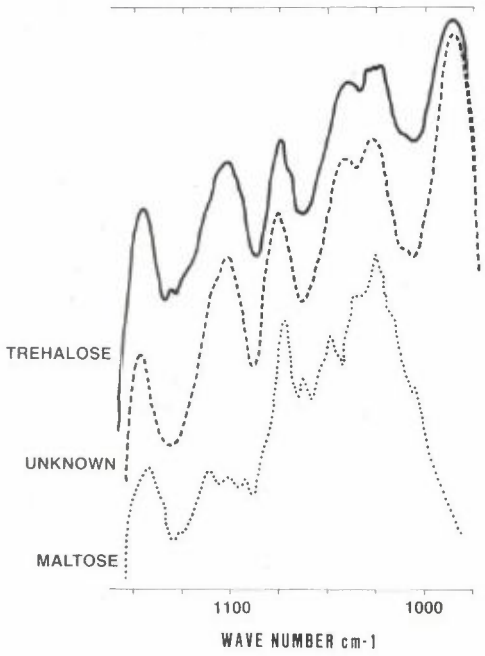

Figure 1. Infrared spectra of trehalose, maltose, and an unknown product from symbiotic algae, strain NC64A.

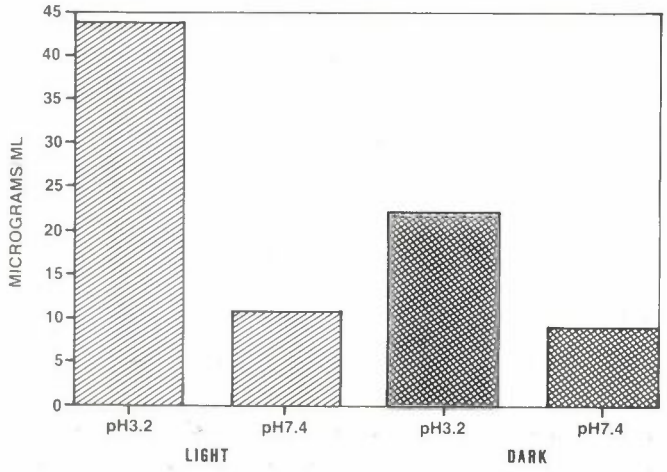

Figure 2. Release of soluble carbohydrate from symbiotic algae, strain NC64A, in light or dark, in media of different pH during 1 hr of incubation. Samples for carbohydrate quantitification (anthrone method) were taken from samples of incubation medium prepared as described under Materials and Methods and reduced to a volume of 1 ml following column chromatography as described.

4. Discussion

Based on prior experience with symbiotic *Chlorella*, the starting assumption was that the material released by the algae probably consisted of small sugar molecules. The data, obtained from a variety of analytical methods, substantiated that assumption. Infrared spectroscopy, of all the tests used, is the most stringent and is widely accepted as being definitive in the identification of molecules such as trehalose. Results from the other analytical methods provided collateral, supportive evidence identifying trehalose as the carbohydrate translocated by these sybionts. As with other sugar translocating *Chlorella*, the trehalose-translocating symbionts released large quantities of the disaccharide *in vitro* when incubated at low pH (Fig. 2). More trehalose was translocated in the light than in dark indicating that trehalose or its precursors may be photosynthetic products. In addition, since trehalose is a non-reducing sugar, it does not participate in forming polysaccharides, hence its production by symbiotic algae cannot be due to hydrolysis of storage polysaccharides or other oligosaccharides.

The precise mechanism of proton activation of translocation (maltose or trehalose) in symbiotic *Chlorella* is unknown, but recent work with brine shrimp larvae has shown that control of trehalose mobilization during encystment and catabolism during hatching, involves pH sensitive hysteretic enzymes (Hand and Carpenter, 1986). For example, at low pH the activity of trehalase is inhibited and trehalose accumulates. Perhaps a similar system operates within symbiotic *Chlorella*, though currently there is no evidence for the presence of a trehalase in NC64A.

Trehalose occurs widely in nature as a storage carbohydrate in bacteria, vascular plants, algae, fungi, and a variety of invertebrates, particularly insects (Elbein, 1974). An interesting property of the molecule is its ability to interact with biological membranes or combine with other moieties to form complexes that interact with membranes. For example, trehalose-membrane interactions have been implicated as an important factor in protecting membranes during cell freezing or desiccation (Crowe et al., 1984; Rudolph and Crowe, 1985; Rudolph et al., 1986).

Trehalose is also a component of sulfatides, the so-called cord factors of tubercular bacteria. These compounds inhibit fusion of lysosomes with phgocytic vesicles (Goren et al., 1976). This inhibition is thought to be critical in the persistence of endoparasitic bacteria. More recently, trehalose has been shown to inhibit fusion between membrane vesicles, findings that seem consistent with the hypothesized role of this molecule in preventing phagolysosome formation. Maltose, a related reducing disaccharide (4-0-(a-

D-glucopyranosyl)-D-glucopyranose), is produced and released by symbiotic *Chlorella*, living in green hydra (*Hydra viridissima*) and green paramecium (*Parmecium bursaria*). Like trehalose, maltose is implicated in the inhibition of host phagolysome formation (Hohman et al., 1982). This inhibition is apparently necessary to prevent the endocellular algal symbionts from being digested by the host's cells. Thus one important (perhaps the most crucial) role of these dissacharides in the symbiosis may be their interactions with host membranes.

In experiments not described here we have seen that the trehalose releasing symbionts can take up endocellular residence in green hydra and establish a stable symbiosis, indicating that trehalose and maltose are interchangeable with respect to the biology of the symbiosis. This apparent interchangeability further suggests that the disaccharides have similar biological properties with respect to the symbiosis.

Cells from the NC64A strain of symbionts (referred to as NC64A P by Mews and Smith, 1982 indicating their origin from Pardy's laboratory) were analyzed by Mews and Smith (1982) who reported that the cells failed to translocate maltose while subcultures from the corresponding NC64A strain (NC64A M) obtained from Leonard Muscatine's UCLA laboratory did. Their published results, based on ^{14}C labeling experiments showed that the NC64A P cells translocated products, but their quantitative analyses failed to show maltose as the translocated sugar. Our data (Fig. 2) show that the symbionts translocate relatively large amounts of carbohydrate and could easily be classified as "high leakers" based on the criteria of Mews and Smith (1982). The failure of Mews and Smith (1982) to quantify the leak product from NC64A P probably results form their technique using amyloglucosidase to hydrolyze maltose with subsequent analysis for glucose. Such a procedure would not detect or measure trehalose. Douglas and Huss (1986), using DNA hybridization techniques suggest that the NC64A-M strain has close affinity with *Chlorella vulgaris*, a free living alga. Citing unpublished DNA hybridization data, Douglas and Huss (1986) suggest that the NC64A P might be *Chlorella kessleri*. Clearly more studies are needed concerning the identify and physiology of these symbionts.

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