PULLULAN ELABORATION, AN INDUCIBLE SYSTEM OF *PULLULARIA PULLULANS*

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1. Introduction

The structure of pullulan, an extracellular α-glucan elaborated by the yeast-like fungus *Pullularia pullulans* has been shown to be predominantly a polymaltotriose linked through α-1,6-bonds on the terminal glucose residues of the trisaccharide [1]. During an examination into the utilization of carbon sources by *P. pullulans* and the organism's ability to elaborate extracellular polysaccharides therefrom, it was observed that pullulan did not appear concomitantly with increase of cell-mass, but began at some point in the middle phase of the organism's growth [2]. Investigations were therefore initiated to delineate the circumstances in which pullulan was elaborated [2]. It is the purpose of this letter to report that pullulan elaboration in *P. pullulans* is not constitutive, but inducible, and may be inhibited by cycloheximide.

2. Materials and methods

Quartermaster strain no. 3092 of *P. pullulans* was maintained and cultured as described previously [2]. Using 2.5% glucose as sole carbon source, cells were grown at 25–27° on a gyrotory shaker at 200 rpm for 48 hr, harvested, and thoroughly washed with water. For carbon substrate uptake and polysaccharide elaboration studies they were resuspended in a medium containing 30 mM phosphate, 17 mM sodium chloride, 0.8 mM magnesium sulphate, 4.5 mM ammonium sulphate, and 14 mM appropriate carbon source containing 0.8 μCi per ml of the ^{14}C-labelled substrate. The medium was adjusted to a cell-density of about 3.5 mg dry cell-weight per ml and a pH of 5.7, an acidity at which pullulan elaboration is fully expressed [2]; and shaken at 25–27° on a gyrotory shaker at 200 rpm. Analyses of carbon substrate uptake and polysaccharide elaboration were performed as described previously, and the chromatographic procedure for the identification of extracellular polysaccharide as pullulan was routinely used [2]. Cycloheximide (B grade, Calbiochem) was used at a final concentration of 8 μg per ml. Labelled compounds were purchased from the New England Nuclear Corporation. All other procedures are to be found in the appropriate legends or described in the text.

3. Results and discussion

An examination of glucose uptake and extracellular polysaccharide production by the starved yeast cells of *P. pullulans* (I, fig. 1) reveals an immediate cellular incorporation of carbon substrate but a delay before the elaboration of pullulan. If starvation was omitted, no delay was observed (II, fig. 1) and it was apparent that polysaccharide elaboration might proceed by an inducible, and not a constitutive, pathway. The addition of cycloheximide, an antibiotic known to inhibit protein synthesis at the ribosomal level [3, 4], to starved Pullularia cells before the addition of glucose (III, fig. 1) eliminates the stimulation of pullulan production, but does not impair the cell's glucose-uptake characteristics. The low level of polymer pro-

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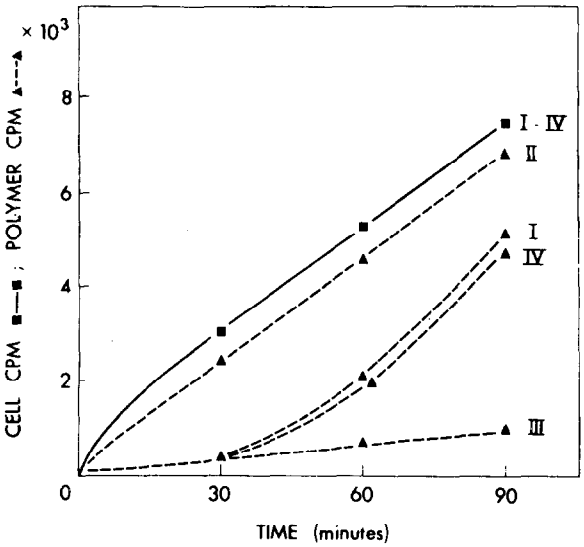

Fig. 1. The uptake of ^{14}C-glucose (■—■—■) and elaboration of polysaccharide (▲—▲—▲) per ml of uptake medium (see text) by washed cells of *P. pullulans* in the yeast phase. Curve II describes glucose utilisation immediately after washing whilst curves I and III-IV represent utilisation after the elapse of 1 hr before glucose addition. Curves I, no cycloheximide; III, cycloheximide added immediately prior to glucose; and IV, cycloheximide added 15 min after glucose supplementation.

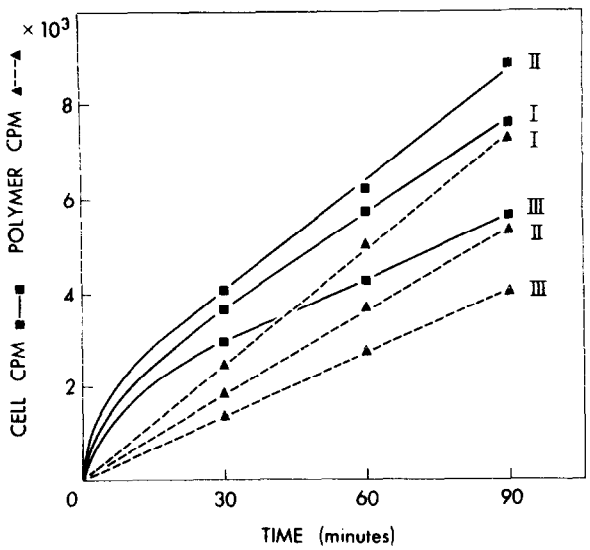

Fig. 2. The uptake of ^{14}C-monosaccharide and elaboration of polysaccharide per ml of uptake medium (see text) by washed cells (3.5 mg dry weight/ml) of *P. pullulans*; for key see fig. 1. Curves I, cells metabolising ^{14}C-glucose that had been exposed to glucose in the induction step (see text); II, cells metabolising ^{14}C-glucose and induced with fructose; and III, cells metabolising ^{14}C-fructose and induced with fructose.

duced by these cells in the presence of antibiotic was 80% pullulan. Treatment of the starved cells with antibiotic 15 min after glucose addition (IV, fig. 1) inhibits neither polysaccharide production or glucose uptake characteristics.

The routine concentration of cycloheximide used in these experiments was 8 μg per ml; however, levels of 0.8 μg per ml could be used to effectively inhibit elaboration. Cells that had been starved for 1 hr, treated with antibiotic at a level of 1 μg per ml for 30 min and then thoroughly washed before resuspension in uptake media containing glucose but no inhibitor, were found to be as effective in glucose uptake and polysaccharide elaboration as control cells that had not been exposed to antibiotic.

To determine the glucose concentration above which effective induction takes place, cells that had been starved for 1 hr in medium containing no carbon substrate were exposed to concentrations of glucose between $0.04–14 \times 10^{-3}$ M for 30 min, and then treated with cycloheximide.

Labelled glucose was added to produce the same final concentration of 14×10^{-3} M and identical specific radioactivities. Analyses of these media showed identical rates of ^{14}C-cellular incorporation but the ability to induce polysaccharide elaboration was not observed below an initial glucose concentration of 2×10^{-4} M (table 1). In a study of the kinetics of carbon substrate uptake by Pullularia, currently under investigation, the apparent K_m for glucose has been shown to be approximately 10^{-4} M. The apparent K_m for 2-deoxyglucose, a sugar which competes with glucose for entry into the cell, is 4×10^{-5} M. These figures indicate that the minimum concentration of glucose which effectively induces extracellular polysaccharide elaboration is probably in excess of that required to saturate the transport system.

Carbon substrates other than glucose were tested for their induction abilities. To cells that had been incubated for 1 hr in the absence of carbon substrate were added glucose, 2-deoxyglucose, fructose, and glycerol to final concentrations of 14 mM. Fructose

Table 1
The stimulation of pullulan elaboration by a range of glucose concentrations at the induction stage (see text).

Glucose concentration (10^{-3} M)	0	0.04	0.20	0.80	4.0	14.0
CPM polymer elaborated per ml per hr × 10^{-3}	1.0	1.0	1.1	2.0	3.7	7.0

and glycerol had been shown to be metabolised by Pullularia [5] and, as indicated above, 2-deoxyglucose is taken up but not utilised. Exposure to these substrates for 1 hr was followed by the addition of cycloheximide, and the cells, washed with antibiotic, were resuspended in standard uptake media in the presence of labelled glucose and cycloheximide. The antibiotic does not inhibit pullulan elaboration of any cells that have already been induced prior to its addition (fig. 1). Polymer elaboration, therefore, following the cells' treatment with antibiotic and measured under standard conditions (described in Materials and methods) with a glucose carbon source, will be a measure of the inducing ability of the substrates described. 2-Deoxyglucose and glycerol failed to induce pullulan elaboration and although fructose was effective, the rate of polysaccharide production was 75% that from induction by glucose. Uptake by the cells was the same in all cases. Cells exposed to fructose in the inducing step, but resuspended in media containing antibiotic and 14 mM fructose in place of glucose in the uptake analysis step, were found to incorporate fructose at 50% the rate of glucose uptake (fig. 2). Polysaccharide elaboration was also found to be reduced to 67% that of the fructose induced glucose metabolising cells, and presumably demonstrates the presence of metabolic controls distributing carbon substrate between cellular requirements and polysaccharide elaboration.

It has been shown that elaboration of pullulan by Pullularia cells in the middle phase of growth, i.e., the cells used in this investigation, may be controlled by the environmental pH [2]. Little or no synthesis occurs at pH 7.5 but adjustment to pH 5.0 produces an immediate elaboration of polysaccharide. Starved cells incubated at pH 7.5 with glucose before the addition of antibiotic, and then adjusted to pH 5.0 again elaborated pullulan. Induction, then, was effected at pH 7.5 even though the cells did not elaborate polysaccharide.

Some aspect, then, of pullulan elaboration has been shown to be inducible, and capable of inhibition by the antibiotic cycloheximide. Furthermore, taking pullulan producing Pullularia cells grown on glucose and subjecting them to an hour's growth on glycerol, an effective carbon source under the conditions of the experiment but incapable of inducing pullulan elaboration; or an hour's starvation, is sufficient to extinguish extracellular polysaccharide production. This is an indication of the lability of some step or steps in the elaboration system. Indeed, no claim is made in this communication to identify which component of the elaboration mechanism, e.g., biosynthesis or the elaboration process itself, is inducible and subject to antibiotic inhibition.

Acknowledgements

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