Which morphological forms of the fungus *Aureobasidium pullulans* are responsible for pullulan production?

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Abstract

Attempts were made to clarify the precise location and possible site of production of the \(\alpha\)-glucan pullulan in different morphological forms of the fungus *Aureobasidium pullulans*. Gold-conjugated pullulanase was used as the specific probe for this purpose. No cell wall pullulan-like material was detected by transmission electron microscopy (TEM) in any morphological form of this fungus, although intracellular electron transparent material bound this probe. When silver enhancement of this gold-conjugated pullulanase probe was used, the data strongly suggested that only swollen cells and chlamydospores, and neither hyphae nor unicellular blastospores, often held responsible for pullulan formation, appeared to produce pullulan-like material.

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1. Introduction

The fungus *Aureobasidium pullulans*, which synthesizes several exopolysaccharides including the \(\alpha\)-glucan pullulan, has a very complex polymorphic life cycle [1, 2], consisting of various unicellular forms and a filamentous mycelium from where individual hyphae often produce these unicells by budding. The unicells described so far include the budding blastospores and single- and multi-celled swollen cells, some of which then convert to melanin-producing chlamydospores [1]. Many factors affect this complex life cycle, with the nitrogen source (both organic and inorganic) and medium pH particularly influential [2]. Equally, pullulan production by *A. pullulans* is sensitive to most of the culture parameters examined, although experimental design has often failed to recognize possible interactions between culture variables (e.g. nitrogen source and culture pH, shear and \(pO_2\)). Such interpretative difficulties are exacerbated by a failure to chemically characterize the polysaccharides produced under different culture conditions in many of these studies [2].

Considerable interest has been shown in trying to associate pullulan production with a particular morphological form in this fungus, since Catley [3] first noted that its formation appeared to coincide with a shift in morphology from mycelial to unicellular forms. Consequently blastospores were held solely responsible for its synthesis [3], a proposal supported by frequent reports of maximum polysaccharide production occurring under culture conditions favoring a unicellular morphology [2, 4]. However, contradictory evidence is plentiful. For example, some studies have shown high total exopolysaccharide yields under culture conditions which support a predominantly mycelial growth form (reviewed by Gibbs and Seviour [2]), although whether pullulan is the sole or even major polysaccharide synthesized under these conditions is generally unspecified.

Furthermore, some reports have suggested that unicellular forms other than blastospores are the major producers of pullulan. Thus, data are available where swollen cell and chlamydospore formation parallels pullulan elaboration [5]. Later, cytochemical evidence [6] was presented indicating chlamydospores were the primary producers of pullulan, a process suggested to involve initial production of exopolysaccharide by the swollen cells, the organization of this into a capsular layer by the derived chlamydo-
spores and finally the release and solubilization of the capsule to form soluble extracellular pullulan. An inverse relationship between intracellular glycogen and extracellular pullulan formation in A. pullulans [7] suggested a link between their production. The cytochemical methods used for detecting glycogen and pullulan relied on staining α-glucol groups of their glucose residues, but it was not clear how these two α-glucans with very similar chemical structures were differentiated by them [6,7]. The assumption was made that all the intracellular α-glucan was glycogen while any extracellular α-glucan was pullulan.

In this study, attempts were undertaken to enhance the specificity of pullulan detection using gold-conjugated pullulanase for its localization. The data confirm that A. pullulans swollen cells and chlamydospores and not blastospores or mycelia are the producers of pullulan-like polysaccharide.

2. Materials and methods

2.1. Organism and culture conditions

A. pullulans (ATCC 9348) was maintained and inocula prepared for shake flask experiments as described previously [8]. The fungus was grown in the chemically defined medium used by Gibbs and Seviour [9] except that glucose and NaNO₃ levels used were 0.25% and 0.78 g l⁻¹ total N. Erlenmeyer flasks (500 ml) containing 150 ml of medium were inoculated and cultures grown at 26°C, 180 rpm on an orbital incubator (Paton Industries, Australia) for 60 h.

2.2. Tagging of pullulanase with colloidal gold particles

Pullulanase (pullulan (1 → 6)-α-glucanohydrolase, EC 3.2.1.41) from Klebsiella pneumoniae (Sigma) was used in these experiments, and conjugated with gold colloid (10 nm diameter, Sigma) using the methodology of Grenier et al. [10] and Roland and Vian [11]. Briefly, the pH of the gold colloid was adjusted using 0.2 M K₂CO₃ or 0.1 M acetic acid to pH 7.6, close to or slightly basic to the isoelectric point of pullulanase (pH 7.5), which was dialyzed first to remove (NH₄)₂SO₄. The minimal amount of pullulanase needed for gold stabilization was determined by diluting it serially in water and then adding 100 μl aliquots of the colloidal gold to the dilution series. After 3–5 min, 100 μl of 10% NaCl was added and any color change from blue (flocculated gold) to red (stabilized gold) was assessed visually. The lowest concentration of pullulanase (as total protein) which prevented this color change was selected as the minimal workable concentration [11], determined here as 4 μg ml⁻¹. A five times excess of pullulanase (200 μg) was used to stabilize 10 ml of the colloidal gold. Three drops of 0.1% (w/v) polyethylene glycol (PEG) 20,000 were added to increase stabilization. This solution was then centrifuged at 32 000 × g at 4°C for 40 min, and the red mobile pool at the bottom of the centrifuge tube containing the gold-labeled enzyme probe was collected and suspended in 0.5 ml 0.2 M acetate buffer, pH 7.6 containing 0.02% PEG 20,000. It was used immediately or stored at 4°C and used within 15 days. Before use it was diluted three times with the acetate buffer.

2.3. Transmission electron microscopy (TEM) of A. pullulans and detection of pullulan

Cells harvested by centrifugation were washed three times in potassium phosphate buffer (50 mM, pH 6.8) and fixed at room temperature for 1 h in 2.5% glutaraldehyde in the phosphate buffer. Cells were then washed three times in the phosphate buffer and dehydrated through an ethanol series of 35, 50, 70, 80, 95% and absolute ethanol for 10 min each, except at 95% and absolute ethanol, where exposure times were 20 min and 1 h, respectively. Cells were infiltrated sequentially in LR white resin mix (ProSciTech), at 1:1, 1:2 and 1:3 for 20 min each before being left overnight in absolute resin under vacuum. Embedding and polymerization were carried out at 55°C overnight in gelatin capsules. Sections (70–90 nm) were cut with a diamond knife and mounted on formvar-coated 300-mesh nickel grids. These grids were floated onto a drop of physiologically buffered saline (PBS; 10 mM, pH 7.4) for 10 min, then transferred onto a drop of gold-conjugated pullulanase and incubated at room temperature for 40 min, washed gently three times with PBS and finally water, before being stained with uranyl acetate and lead citrate. Control sections were stained in the same manner except unconjugated gold colloid was used. These were examined with a JEOL JEM 100CX transmission electron microscope (TEM) at 80 kV and images were captured on Kodak EM film 4489.

2.4. Silver intensification of gold-labeled pullulanase for light microscopy

Cells were removed from shake cultures, directly smeared onto glass slides and allowed to air dry. After gentle washing with PBS for 10 min, an aliquot of the gold–pullulanase complex (50 μl in 450 μl PBS, prepared as in Section 2.2, but using 20 nm colloidal gold), sufficient to flood the smear was added and the slide incubated for 1 h at room temperature in a moist chamber. It was then gently washed three times in Milli-Q water and dried. Smears were then flooded with silver stain solution (Sigma Technical Bulletin No. SE-1) for 5–10 min or until the solution color changed from clear to dark, before being gently washed again. Smears were then exposed to sodium thiosulfate (2.5% w/v) for 2–3 min to stop the reaction. Slides were air dried before being counterstained with toluidine blue and mounted in histoclear. A control was included, where the pullulanase–gold complex was replaced by unconjugated gold colloid. Slides were viewed...
by light microscopy with a Nikon Eclipse E800 microscope using Nomarski interference optics, and images captured with a Nikon digital camera (DXM 1200).

3. Results and discussion

3.1. Properties of cultures of *A. pullulans* used in this study

Culture morphology was predominantly unicellular at the sampling time, consisting of a mixture of blastospores, swollen cells and chlamydospores. A few germinated swollen cells with short germ tubes and individual hyphae were also present. Extracellular polysaccharide recovered by ethanol precipitation was shown to consist of pullulan by its susceptibility to digestion and release of reducing sugars after incubation with pullulanase [8].

3.2. Localization of pullulan-like polysaccharide associated with cells of *A. pullulans*

When thin sections of cells exposed to the gold-conjugated pullulanase were examined by TEM, no gold-conjugated pullulanase was ever seen associated with the cell wall or cell surface in any of the cells, and no evidence was obtained with it to support the idea that pullulan-like polysaccharide might be organized in a capsular layer around them (Fig. 1a,b). It was not possible to state with certainty which sectioned morphological forms were viewed here but the original culture contained representatives of all the unicellular forms, and extracellular pullulan was being produced.

Instead, the gold-conjugated pullulanase was always clearly associated with intracellular granules of electron translucent material (Fig. 1a,b), suggesting that a material...
capable of binding with pullulanase is deposited intracellularly. Either this intracellular material is pullulan, or another \( \alpha \)-glucan containing \( (1 \rightarrow 6) \- \alpha \)-glucosidic linkages binding to pullulanase. Some cells (arrowed) were seen which appeared not to contain it (Fig. 1a). Although the preferred substrate for this pullulanase is pullulan, it is also capable of hydrolyzing the \( (1 \rightarrow 6) \- \alpha \)-glucosidic linkages in both glycogen and amylopectin [12].

It is possible that any extracellular pullulan-like polysaccharide initially present was removed from the surfaces of the cells during the TEM preparation protocol, and both Simon et al. [6] and Takeo et al. [13] have implied that the capsular polysaccharide layer in \( \text{A. pullulans} \) is very readily detached. Consequently, silver enhancement was used to enlarge the colloidal gold particles so that whole unashed cells could be examined by light microscopy to see if a surface-associated pullulan-like polysaccharide was restricted in its occurrence to a particular morphological form. The results (Fig. 1c–f) clearly show this to be the case. Only the multi-celled chlamydospores and swollen cells were coated with silver grains (Fig. 1c,d) reflecting the specific tagging with the gold-conjugated pullulanase and hence the likely presence of pullulan-like polysaccharide on their surfaces. None of the blastospores (Fig. 1d), germ tubes arising from swollen cells (Fig. 1e) or hyphae (Fig. 1f) showed this silver deposition, consistent with them lacking any pullulan-like associated polysaccharide. These results support the earlier data of Simon et al. [6] that swollen cells and chlamydospores and not blastospores, as once thought [2], synthesize pullulan. They also agree with Simon et al. [6] who suggest that whatever polysaccharide might be synthesized by hyphal forms of \( \text{A. pullulans} \), it is not pullulan-like (Fig. 1f). Whether the intracellular pullulanase-tagged material is a glycogen-like polymer serving as a precursor for extracellular pullulan formation, as proposed by Simon et al. [6,7], is not clear from these studies because the chemical nature of this material could not be satisfactorily elucidated (data not shown), but these data do raise interesting questions about possible relationships between their formation.

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