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Penicillin-binding proteins in the cyanelles of *Cyanophora paradoxa*, a eukaryotic photoautotroph sensitive to β-lactam antibiotics

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Cyanophora paradoxa is a eukaryotic protist sensitive to β-lactam antibiotics, most likely as a consequence of the binding of the drugs to a set of 7 proteins, analogous to eubacterial penicillin-binding proteins (PBPs), located in the envelope of the *C. paradoxa* photosynthetic organelles, the cyanelles.

Penicillin-binding protein; Cyanelle; β-Lactam; *(Cyanophora paradoxa)*

1. INTRODUCTION

Cyanophora paradoxa, a strictly photoautotrophic protist, harbours cyanelles fulfilling the functions of chloroplasts. In morphology and pigment composition cyanelles resemble unicellular cyanobacteria, whereas the cyanellar genome is in the size range of chloroplast DNAs [1]. Isolated cyanelles are stable in hypotonic solution but lyse readily in the presence of lysozyme [2]. Analysis of the cyanelle envelope revealed the presence of a peptidoglycan similar to that of Gram-negative eubacteria [3,4]. The uniqueness of a murein sacculus enclosing an organelle in a eukaryotic cell, stimulated us to investigate further its relevance to the growth and division of the cyanelle. Based on the assumption that murein biosynthesis in cyanelles would be a β-lactam-sensitive process, as in eubacteria, we studied the effects of these antibiotics on the *Cyanophora*/cyanelle system as an initial experimental approach to the understanding of the role of cyanelle murein.

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2. MATERIALS AND METHODS

C. paradoxa LB 555 UTEX was grown as described [5]. Sensitivity of *C. paradoxa* to β-lactams was assessed by incubating 2-ml cultures supplemented with serial dilutions of the corresponding β-lactam for 48 h. Discoloration indicated cell death. Cyanelles were prepared as in [5] and further purified by centrifugation (10 min, 2500 × *g*, 4°C) on a step gradient consisting of 5 ml of 90% (v/v) Percoll and 10 ml of 72% Percoll in 50 mM phosphate buffer, pH 7. Cyanelles were suspended to an appropriate density in 50 mM phosphate buffer, pH 7. All solutions were supplemented with the protease inhibitors PMSF, TPCK and TLCK at 2 mM final concentration. For β-lactam-binding assays, 60 μl of cyanelle suspension (3×10^8 cyanelles/ml) were incubated for 15 min at 26°C with *N*-(3-(4-hydroxy-5-[^{125}I]iodophenyl)propionyl)ampicillin (^{125}I-ampicillin) (2000 Ci/mmol) at a final activity of

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100 μCi/ml [6]. Afterwards, the samples were centrifuged (30 s, 10000 × *g*), the pellets were resuspended in an equal volume of water, diluted 4:1 in 160 mM Tris-HCl, pH 6.8, 20% (w/v) SDS, 10% (v/v) β-mercaptoethanol, boiled for 10 min, and upon centrifugation (10 min, 10000 × *g*), the supernatants were subjected to SDS-PAGE on 8% (w/v) acrylamide slab gels [7]. Labeled proteins were identified by autoradiography and quantified by densitometry. Competition experiments to study the binding of unlabeled β-lactams to cyanelles were performed as described [8].

3. RESULTS AND DISCUSSION

To define the physiological relevance for *C. paradoxa* of the murein layer in the cyanelle envelope, a number of β-lactam antibiotics were tested with cultures of *C. paradoxa*. All the β-lactams tested were in most cases lethal to *C. paradoxa* at concentrations in the 1–25 μg/ml range (table 1), which compares well with published data for eubacteria [9].

Fig.1 shows the effect of ampicillin (50 μg/ml) on the growth of a culture of *C. paradoxa*. Immediately after addition of the drug, the cell number stopped increasing, remaining constant for about 24 h, when a rapid decline began. Microscopic observation suggested that longitudinal division of *C. paradoxa* cells was impeded by blockade of cyanelle division. The daughter cells being unable to share the single non-dividing cyanelle break apart, releasing dumb-bell shaped cyanelles (fig.1). In cultures held in the dark no decrease in cell number was observed during the experimental period.

Assuming that the last stages of murein biosynthesis in cyanelles were mediated by penicillin-binding proteins (PBPs) as in eubacteria, we attempted their detection by means of binding assays with ^{125}I-ampicillin as probe. At the highest concentration of antibiotic used (80 nM) a total of 10 labeled proteins were identified in isolated cyanelles (fig.2, lane 6). Binding of ^{125}I-ampicillin to 3 of them could not be prevented by preincubation of cyanelles with ampicillin (100 μg/ml) (fig.2, lane 7), and they were rejected as artifactual. The remaining 7 proteins were considered to be genuine

Table 1

Interaction of β-lactam antibiotics with *C. paradoxa*

β-Lactam	MIC[a] (μg/ml)	ID_{90}[b] (μg/ml) for PBP[c]					
		1	2	3	4	5	7
Amoxicillin	1	–	1	0.1	1	1	10
Ampicillin	5	0.1	1	1	1	10	10
Azthreonam	100	–	10	10	10	1000	>1000
Benzylpenicillin	1	–	1	0.1	0.1	1	10
Carbenicillin	1	1	2	1	1	1	100
Cefsulodin	100	10	100	10	10	100	1000
Cephalexin	25	–	1	1	1	20	>100
Imipenam	0.5	0.5	1	0.1	0.1	1	0.1
Mecillinam	25	–	10	10	100	>100	>100

[a] Minimal inhibitory concentration

[b] Concentration of β-lactam required to reduce ^{125}I-ampicillin binding to a given PBP by 90%

[c] PBP 6 could not be properly quantified because of high background fogging of the corresponding area on the autoradiograms

Minimal inhibitory concentrations were calculated as described in section 2. The residual binding of ^{125}I-ampicillin to each of the PBPs was measured after preincubation of cyanelles with increasing concentrations of unlabeled β-lactams for 15 min at 26°C

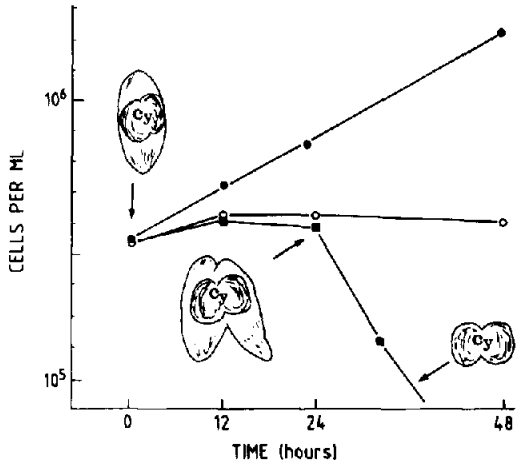

Fig.1. Effect of ampicillin on the growth and morphology of *C. paradoxa*. An early log-phase culture of *C. paradoxa*, growing at 26°C with a photoperiod of 20 h light–4 h darkness, was divided into three equal subcultures. At time 0 min one of them (■) was treated with ampicillin (50 μg/ml), the second wrapped in aluminum foil to be kept in darkness (○) and the third one used as untreated control (●). The subcultures were further incubated under the same conditions and samples were withdrawn periodically to measure cell number in a Neubauer chamber and for microscopical observation. Drawings schematically represent the morphology of *C. paradoxa* cells and cyanelles (cy) observed in ampicillin-treated cultures at the times indicated by the arrows.

PBPs and numbered in order of increasing electrophoretic mobility. The apparent M_r values estimated by comparison with the PBPs of *E. coli* [8] were 110000, 100000, 84000, 72000, 54000, 40000 and 31000, respectively. A binding experiment performed with soluble and particulate subfractions, obtained by differential centrifugation of cyanelles broken by sonication, indicated that the PBPs were associated quantitatively to the particulate fraction (not shown). However, in purified envelopes the PBPs became very susceptible to proteolytic degradation, making the use of intact cyanelles more convenient. The kinetics of binding (fig.2, lanes 1–6) indicated that PBPs 2, 3 and 4 had a high affinity to the β-lactam probe, reaching saturation under the conditions adopted with apparent half-saturating concentrations of 2, 7 and 6 nM, respectively.

The stability of the ^{125}I-ampicillin-PBP complexes was estimated by binding experiments in which, upon labeling with ^{125}I-ampicillin, cyanelles were washed by centrifugation, resuspended in buffer supplemented with unlabeled ampicillin (200 μg/ml), and incubated for up to 2 h at 26°C prior to denaturation. While complexes between ^{125}I-ampicillin and PBPs 2, 3, 4 and 5 were virtually stable with no apparent decay after 2 h, PBP 7 formed short-lived complexes (half-life <20 min), a characteristic shared with PBPs 5/6 of *E. coli* [8].

To assess the functionality of the individual PBPs, the pattern of binding of a number of β-lactams lethal to *C. paradoxa* was established by means of competition experiments. The results are summarized in table 1. Although differential binding patterns were clearly observed, and the magnitudes of the concentrations preventing binding of the radioactive probe to the PBPs correlated reasonably with the lethal concentration of the antibiotics, their specificity of binding was insufficient to answer this question. Nevertheless, our data suggest that simultaneous inhibition of PBPs 2 and 3 might be enough to impair proliferation of cyanelles to an extent lethal to *C. paradoxa*.

To exert the reported effects, β-lactams should first permeate into the *C. paradoxa* cytoplasm, where environmental conditions could modify their interaction with the cyanelle. Therefore, the binding of ^{125}I-ampicillin to intact *C. paradoxa* cells was studied (fig.2, lane 8). The patterns of binding to whole cells and to cyanelles were essentially the same, indicating that all PBPs in the cyanelle envelope can react with ^{125}I-ampicillin in the cytoplasm. The lack of additional binding proteins in whole cells further supports the role of cyanelle PBPs as the lethal targets for β-lactams in *C. paradoxa*.

The copy number per cyanelle for each PBP was estimated by measuring the radioactivity associated to each band in the gels, upon electrophoresis of cyanelles labeled (15 min, 26°C) with ^{125}I-ampicillin at 80 nM. Reliable data could be obtained solely for PBPs 2, 3 and 4 (100, 12 and 140 copies/cyanelle, respectively), the only PBPs saturated under our binding conditions. Nevertheless, the values for PBPs 1, 5 and 7 (10, 70 and 130 copies/cyanelle, respectively) do provide an idea of their minimal numbers. Although limited, our results indicate that PBPs are present in

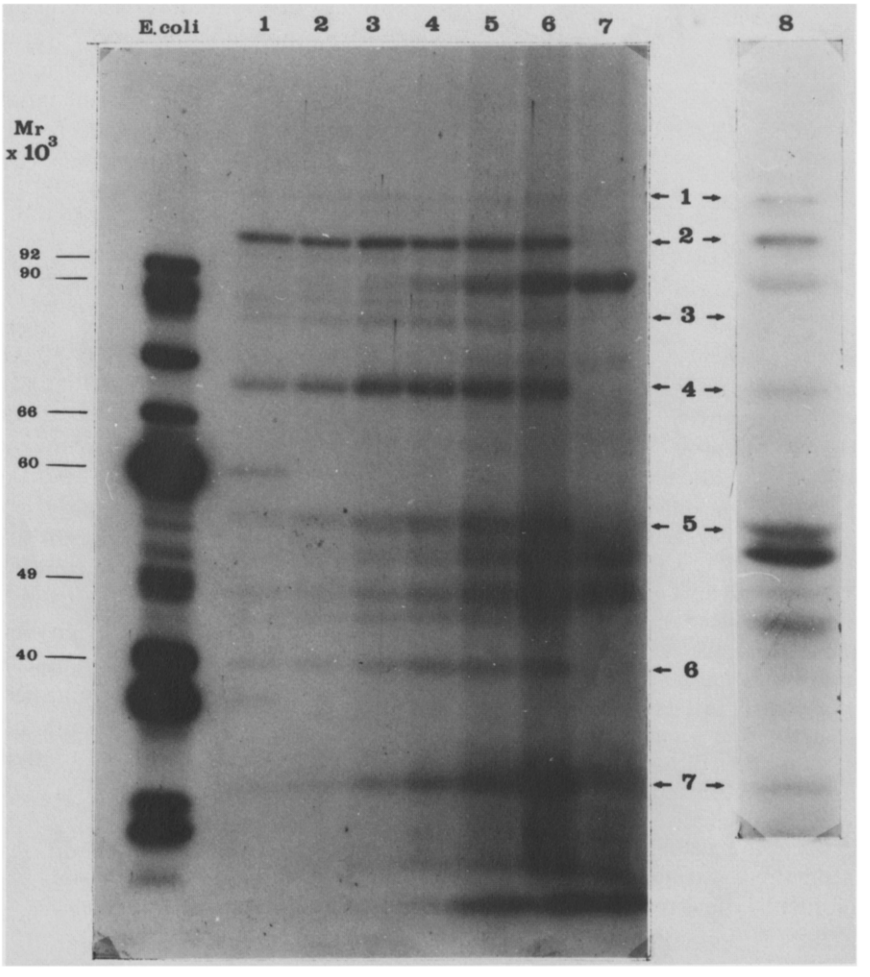

Fig.2. Detection of penicillin-binding proteins in cyanelles of *C. paradoxa*. To label PBPs in isolated cyanelles (lanes 1–7), 60-μl aliquots of cyanelle suspension were incubated with increasing concentrations of ^{125}I-ampicillin (2.5, 5, 10, 20, 40, 80 nM for lanes 1–6, respectively) as indicated in section 2. The sample in lane 7 was preincubated with ampicillin (100 μg/ml) for 10 min before addition of ^{125}I-ampicillin at 80 nM final concentration. Labeling to intact cells (lane 8) was performed incubating a 60 μl aliquot of a suspension of *C. paradoxa* cells (approx. 10^8 cells/ml) with ^{125}I-ampicillin at 80 nM for 20 min at 26°C. Labeling was stopped by addition of ampicillin (200 μg/ml), cells were sedimented by centrifugation (30 s, 10000 × g), resuspended in 60 μl water and further treated as described for isolated cyanelles. The PBPs of *E. coli*, labeled with ^{125}I-ampicillin as described [10], were used as M_r standards (lane *E. coli*) [8].

cyanelles and in *E. coli* in comparable numbers [8].

The information presented here supports the idea that cyanelles have enzymes similar to eubacterial PBPs, whose inhibition by β-lactams impairs division of the organelle which, in turn, blocks division of the host cell itself. The presence of PBPs, so far undetected in eukaryotic cells, in *C. paradoxa* constitutes a strong argument in favor of the evolution of chloroplasts from endosymbiotic cyanobacteria.

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REFERENCES

[1] Bohnert, H.J., Crouse, E.J., Pouyet, J., Mucke, H. and Löffelhardt, W. (1982) Eur. J. Biochem. 126, 381–388.
[2] Schenk, H.E.A. (1970) Z. Naturforsch. 25B, 656–661.
[3] Aitken, A. and Stanier, R.Y. (1979) J. Gen. Microbiol. 112, 219–223.
[4] Giddings, J.T. jr, Wassman, C. and Staehelin, L.A. (1983) Plant. Physiol. 71, 409–419.
[5] Mucke, H., Löffelhardt, W. and Bohnert, H.J. (1980) FEBS Lett. 111, 347–352.
[6] Schwarz, U., Seeger, K., Wengenmayer, F. and Streker, H. (1981) FEMS Microbiol. Lett. 10, 107–109.
[7] Laemmli, U.K. and Favre, M. (1973) J. Mol. Biol. 80, 575–599.
[8] Spratt, B.G. (1977) Eur. J. Biochem. 72, 341–352.
[9] Noguchi, H., Matsuhashi, M. and Mitsuhashi, S. (1979) Eur. J. Biochem. 100, 41–49.
[10] Rojo, F., Ayala, J.A., De la Rosa, E.J., De Pedro, M.A., Aran, V., Berenguer, J. and Vazquez, D. (1984) J. Antibiotics 37, 387–393.