Penicillin-binding proteins of protoplast and sporoplast membranes of Streptomyces griseus strains

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Abstract. Membrane-bound penicillin-binding proteins (PBPs) of two Streptomyces griseus strains that sporulate well in liquid and solid medium have been investigated during the course of their life-cycle. The PBP patterns were analyzed by sodium dodecylsulphate polyacrylamide-gel electrophoresis and fluorography. One strain (No. 45 H) has only a single band (mol wt: 27,000) in early log phase, and two additional PBPs of higher mol wt (69,000 and 80,000) in the late log phase. The other strain (No. 2682) possessed two bands with mol wts 27,000 and 38,000 which did not change during its vegetative phase. In strain No. 2682, a new PBP with a mol wt of 58,000 appeared in spore membranes while one of those (mol wt 38,000) present in mycelial membranes disappeared. Our results suggest that appearance of the new PBP in the spore may be associated with the sporulation process. The major PBP band (mol wt: 27,000) present in all stages of the life cycle of these strains, may be characteristic of S. griseus while the other PBPs reflect certain stages of the life cycle. A new method was developed for the production of spor protoplasts by consecutive enzymatic treatments.

Key words: Penicillin-binding proteins – Protoplast – Sporoplast – Streptomyces griseus

Bacterial cells contain multiple penicillin-binding proteins (PBPs) which catalyze terminal steps of cell wall synthesis. Studies on Streptomyces strains show that they usually possess three or more penicillin-binding proteins (Ogawara and Horikawa 1980; Horikawa et al. 1980; Dusart et al. 1981). Comparing the PBP patterns of β-lactam producing and β-lactam-non-producing Streptomyces strains has revealed marked differences in the number and affinity for penicillin (Nakazawa et al. 1981). Investigation of age-dependent changes has shown that the PBP patterns are also affected by the medium composition (Nakazawa et al. 1981). Two new PBP bands appear in sporulating cells of Bacillus subtilis and their association is a consequence of sporulation events (Buchanan and Sowell 1983; Neyman and Buchanan 1985). Little is known, however, about the alteration of the PBPs during the sporulation of Streptomyces.

We examined the PBP patterns of the vegetative mycelium at different ages of Streptomyces griseus cultures as well as those of the spores. Some strains of Streptomyces griseus can sporulate well in submerged culture under appropriate nutritional and environmental conditions resulting in phase-bright spores (Kendrick and Ensign 1983). Differences were found in PBP patterns and a new method was developed for obtaining sporoplasts and spore membranes by a combined enzymatic treatment of the spores.

Material and methods

Streptomyces griseus (No. 45 H), a streptomycin (SM) non-producer (Szabo et al. 1961) and No. 2682, a SM producer wild type strain were used in this study. The strain No. 45 H was derived from a SM producing and poorly sporulating strain No. 52-1 by mutation (Szabo et al. 1961). The strain No. 2682 was a generous gift of Dr. J. C. Ensign, University of Wisconsin, Department of Bacteriology, Madison, WI, USA. Strains were grown in synthetic liquid medium (Leyh-Bouille et al. 1971) containing 2% v/v glycerol, 0.5% NaCl, 0.1% CaCO3, 0.1% MgSO4, 1% K2HPO4, 0.1% FeSO4 and 0.03% (w/v of each) casein hydrolysate (kindly provided by the Biogal Pharmaceutical Works, Debrecen). Cultures were incubated at 27°C with a shaking frequency of 250 rpm in a rotatory shaker (New Brunswick Scientific Co. Model No. G-25).

Growth and morphological changes during the life cycle were determined as described previously (Szabó et al. 1961).

Antibiotics

Para-[3H]-benzylpenicillin ethylpiperidinium salt with a specific activity of 773.3 GBq/mmol was purchased from the Institute of Isotopes, Hungarian Academy of Sciences, Budapest, Hungary. Potassium salt of benzylpenicillin was obtained from Biogal Pharmaceutical Works, Debrecen, Hungary.

Isolation of the cytoplasmic membrane

A method introduced by Dusart et al. (1973) was applied. Cells harvested at appropriate age were washed twice with distilled water and once with 0.1 M Tris – HCl buffer (pH 7.5), then resuspended in the same buffer containing 25%
(w/v) of sucrose and 500 µg/ml of egg lysozyme (Serva, 3x crystallized), and incubated at 5°C overnight. After at least 90% of the cells had been converted to protoplasts, they were centrifuged at 27,000 x g for 20 min at 4°C. Protoplasts were resuspended in 0.1 M Tris-HCl buffer (pH 7.5) containing 0.01 M MgCl₂ and 7.5 mg DNase, and incubated for 5 h at room temperature. Intact cells were removed by sedimenting at 1,500 x g for 10 min at 4°C. The supernate containing the membranes was centrifuged at 36,000 x g for 30 min at 4°C and membranes were resuspended in 14 mM phosphate buffer (pH 7.3). The membrane fraction was stored at -20°C without loss of D,D-carboxypeptidase-transpeptidase activity. Protein concentration was measured by the Lowry method (Lowry et al. 1951).

**Isolation of sporoplasts and spore-membrane**

Spores of cells grown in glycerol-casein liquid medium were harvested at 72 h. They were washed once with water and once with 0.1 M Tris-HCl buffer (pH 7.5) and then ultrasonicated with a Braunsonic 300S sonifier by 5 pulses of 30 s in an ice bath to destroy the contaminating mycelial parts. After centrifugation at 3,000 x g for 10 min at 5°C the spores were sedimented and then resuspended in the above buffer containing 500 µg/ml of trypsin (Serva, bovine pancrease, 2x cryst.). They were incubated for 5 h at 5°C, then sedimented at 3,000 x g for 10 min at 5°C and washed with cold water. Incubation was continued in 0.1 M Tris-HCl buffer (pH 7.5) containing 25% (w/v) sucrose and 1 mg/ml lysozyme (egg white, 3x cryst.) at 5°C overnight. Membranes were then prepared as described above for the mycelial membranes. Spore membranes were stored at -20°C.

**Labelling of the PBPs with radioactive penicillin**

Membranes (containing about 200 µg protein) were incubated with 50 µM of [³H]-benzylpenicillin (specific activity 773.3 GBq/mmol) in a total volume of 21 µl at 37°C for 10 min and then supplemented with excess non-radioactive benzylpenicillin to a final concentration of 10 mM. Twenty-five microliters of denaturating buffer was added containing 10% (w/v) glycerol, 0.5 M Tris-HCl, 2% (w/v) SDS, 0.01% (w/v) bromphenol blue, 10% (v/v) 2-mercaptoethanol was added and the suspension was boiled for 2 min. The samples were subjected to sodium-dodecyl-sulphate (SDS)-polyacrylamide-gel electrophoresis (PAGE).

**SDS-PAGE and fluorography**

The SDS-PAGE procedure was carried out as described by Laemmli and Favre (1973) with an acrylamide content of 4% and 10% (w/v of each) in the stacking and running gels, respectively. After separation of proteins at a constant 360 V the gels were fixed in a solution containing 5% (v/v) methanol and 7.5% (v/v) acetic acid. Gels were placed in dimethylsulfoxide to remove water, followed by 2 h in a 20% (w/v) solution of 2,5-diphenyloxazol in dimethylsulfoxide. Following rehydration in water, gels were dried and exposed to Medifort RP X-ray film for 7-10 days at -70°C (Bonner and Laskey 1974). The band intensities were visually evaluated.

**Electron microscopy**

Cells were harvested by centrifugation, washed once in distilled water and fixed in 4.5% glutaraldehyde in 0.1 M
sodium cacodylate buffer pH 7.2 at 0°C overnight. After washing with the cacodylate buffer for 30 min the samples were postfixed in a mixture of 0.5% OsO₄ and 2.5% K₂Cr₂O₇ in the cacodylate buffer 2 h in 0°C, poststained in 1% uranylacetate, dehydrated in a graded ethanol series and embedded in Epon 812. Thin sections were cut with a diamond knife on a LKB Ultrotome and examined in a Philips EM 300 without further staining.

Results

PBP patterns of the vegetative mycelia

The PBP patterns of membranes isolated from early and late logarithmic phase cultures were compared (Fig. 1). In the early log phase, strain No. 45 H possessed one PBP with a molecular weight of 27,000. In the late log phase, two additional PBP showed up with mol wts of 69,000 and 80,000.

Examination of the PBPs from vegetative mycelia of strain No. 2682 showed two bands (mol wts 27,000 and 38,000) in the early or the late log phase.

Preparation of sporoplasts from strain No. 2682 and detection of their PBPs

Sporules from the strain No. 2682 were obtained from a liquid medium containing 0.03% (w/v) of casein hydrolysate, and mycelial contaminants were removed. Formation of sporoplasts was followed by phase-contrast and electron microscopy. Ultrathin sections of spores and of sporoplasts are shown in Fig. 2.

Although we cannot exclude the possibility that some remnants of the cell wall remained attached to the sporoplasts, our main purpose was to obtain membranes, and the procedure used met this demand. It is of interest that spores incubated under the same conditions but not exposed to the enzymes (trypsin, lysozyme) did not germinate.

The fluorographic patterns of membranes from mycelia and from spores showed significant differences (Fig. 3). Both contained a PBP with a mol wt of 27,000. The second and minor PBP of mol wt 38,000 found in membranes of the mycelia, was absent from spore membranes but these contained an additional one with a mol wt of 58,000. As an additional control, membranes were isolated from spores previously killed by γ-ray treatment. The PBP pattern exhibited the same extra band at mol wt of 58,000. The possibility that this PBP was from membranes of spores germinating during the enzymatic treatments is thus excluded.

It is noteworthy that the PBP with a mol wt of 27,000 is present in both of the strains examined and at all stages of the life cycle.

Further evidence of forming protoplasts from spores is their osmotic fragility. When we kept them in water they burst and less than 5% remained alive measuring by viable counts.

Discussion

A considerable variation was found in PBP patterns of Streptomyces griseus strains. The major PBP component of mol wt of 27,000 was present in all stages of the life cycle, while additional bands appeared in later stages, presumably in connection with sporulation process.

Although Streptomyces spores are resistant to such lytic enzymes as lysozyme and muramidase, digestion of the spore cell wall was brought about by employing a trypsin treatment prior to addition of lysozyme. Electron microscopic observations showed that sporoplasts were sometimes formed directly by trypsin treatment. While the rationale for this behaviour is unknown, it is possible that “triggering” of autolytic enzyme activity might be involved. Furthermore, as the electron micrographs showed a part of outer layer of the spore cell wall not susceptible to lysozyme was removed by trypsin.

In view of the difference in the structure between the spore cell wall and that of the mycelium, it can be presumed that one or more penicillin sensitive enzyme(s) present in spore forms are responsible for biosynthesis of the spore cell wall.

In certain bacterial strains (e.g. Bacillus subtilis, some Streptomyces strains) PBP patterns are dependent on the growth cycle (Buchanan and Sowell 1983, Nakazawa et al. 1981). The strain No. 45 H possess a relatively short life cycle, thus at the age of 48 h it is in a pre-spore form. The strain No. 2682 is in vegetative form at the same age, and spores are formed later, at 72 h. The appearance of additional bands (mol wts of 69,000 and 80,000; and 58,000 in the strains 45 H and 2682, respectively) may be correlated with formation of spore cell wall.

A new method elaborated for the preparation of Streptomyces spore membranes (Barabás et al. 1986) provided the opportunity to detect additional PBPs in spores. This is probably more suitable for preserving large active membrane-bound enzymes then the procedure which involves mechanical disruption of the spores (Quiros et al.
1986) since the latter method can destroy some large molecules as well as the structure of membrane by its shearing effect.

References

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