Ph. D.THESIS

STUDY OF THE ROLE OF BETA-LACTAMASE ENZYME AND THE EFFECT OF BETA-LACTAM ANTIBIOTICS IN- *STREPTOMYCES GRISEUS* NRRL B-2682

Eleonóra Deák

Supervisor:
Dr. Penyige András

and

Dr. Szabó István

University of Debrecen
Medical and Health Science Center, Faculty of Medicine
Institute of Humangenetics

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INTRODUCTION

Importance of Streptomyces

Streptomyces are Gram-positive, soil bacteria, known for their elaborate life cycle. S. griseus has three important characteristic, which make it different from the other *Streptomyces* species: 1. The aminoglycoside antibiotic, streptomycin was discovered in this strain. 2. Uniquely this bacterium forms spores in liquid medium, for this reason the morphological differentiation of this species is easier to study. 3. *S. griseus* also produces a small molecular weight diffusible autoregulator, the A-factor, this extracellular molecule is necessary for spore formation and streptomycin production too.

The life cycle of Streptomyces is different whether this bacterium is grown on solid or liquid medium. On solid medium spores of *Streptomyces griseus* germinate to produce a complex network of apically growing hyphae that forms the substrate mycelium (or vegetative mycelium), which later develops into aerial mycelium. Most Streptomyces species can be grown in liquid medium under proper oxygenation. Under this condition the life cycle is different, because the spores develop directly from the reproductive mycelium.

β-lactame antibiotics

β-lactame antibiotics are named after its β-lactame ring, which is formed by 3 C and 1 N atom. Besides the penicillins, cephalosporins, monobactams and carbapenems belong to the β-lactame antibiotic family.

The penicillins are the acylated product of 6-aminopenicillinic acids. For its biological activities of penicillins the β-lactame ring is responsible. Resistance against this antibiotic most frequently occurs through the production of b-lactamase enzyme by bacteria. This enzyme is capable to break up the β-lactame ring, making the antibiotic ineffective.

The lethal effect of β-lactame antibiotics is a very complex process. These antibiotics have a bactericide and also bacteriolytical effect. The bacterium will dye soon after the treatment with penicillin, while cell lysis will occur after a significant lag-period. Effectivity of penicillins depends on the growth phase of the bacteria too. Only those bacteria will be killed which are in the exponential growth phase. As soon as the bacterium enters the stationary phase, bacterium is able to survive penicillin treatment.

The basis for the therapeutic, bactericide effect of penicillins is that this antibiotic inhibits the carboxypeptidase enzyme, which catalysis the last step of the bacterial cell wall, peptidoglicane biosynthesis.

Lysis of bacteria

More than 70 years of after its discovery, penicillin's bacteriolytical effect is still not completely understood. The lytic effect of penicillin and related molecules can be explained by two hypotheses. Based on the first hypothesis β-lactame antibiotics disturb the normal function of the endogen autolysins which play role in the cell wall metabolism. The integrity of the peptidoglycan breaks up, and this leads eventually to the lysis of the bacteria. The other hypothesis uses the results of bacteriaophage generated cell lysis. According to this theory holing-antiholin proteins regulate autolysis. It was recently published that in E. coli infected with delta phage, the depolarisation of the membrane potential resultsed in the assembly of deltaS holin proteins in the cell membrane. These holin proteins form wholes in the cell membrane, and through this hole the autolytic enzymes can move across and easily access their substrates, resulting in lysis of the cell wall. Proteins similar to holins have been described in bacteria.

Characteristics of β-lactamases

β-lactamase enzymes can be found both in the Gram-positive and Gram-negative bacteria. The only known function of these enzymes is the hydrolysis of the B-lactame ring, resulting in the inactivation of the antibiotic. Most known β-lactamase (A, C and D class) contains serine in its active site. When β-lactamase reacts with its substrate, an acil-enzyme intermedier forms, which is hydrolysed quickly, and the inactive penicilloic acid will form. This reaction is responsible for antibiotic resistance against β-lactam antibiotics in most pathogenic bacteria.

Tipically β-lactamases are extracellular proteins, as they are either excreted into the growth medium in Gram-positive bacteria or they are accumulated in the periplasmic space in Gram-negative bacteria.

Most Streptomyces species are especially resistant high concentration of penicillin. Interestingly, in these species the role of β-lactamase enzymes in the development of resistance is debated, because there is no correlation between the level of enzyme production and the degree of antibiotic resistance. In addition, in these species the protection against the

β-lactam antibiotics are most likely not due to the β-lactamase production, instead the presence of PBP molecules, which has very little affinity towards β-lactam molecules.

However, the physiological role of β-lactamases, in non-pathogenic and in bacteria incapable of β-lactam production, (like *Streptomyces*) is still not completely understood. Until the 1990s it was assumed, that β-lactamases are not necessary for the peptidoglycan metabolism. However, based on the latest discoveries it is suggested that β-lactamase enzymes might play a role in cell wall metabolism of bacteria.

OBJECTIVES

1. We studied the production and localization of β-lactamase enzyme in *Streptomyces griseus* NRRL B-2682 and in its nonsporulating spontaneous mutant *spo1*$^-$ strain.
2. We have isolated the membrane-bound and the extracellular β-lactamase enzyme from the wild type *S. griseus* and the membrane-bound form from the mutant *spo1*$^-$ strain. The N-terminal sequence of the extracellular enzyme was also determined.
3. We have studied the possible physiological role of β-lactamase enzyme in *S. griseus* strain, which do not produce β-lactam antibiotics, however it is highly resistant to β-lactam compounds.
4. We have developed a method to study cell wall autolysis induced by β-lactams and ionophores in our strain.
5. Using a $\Delta\Psi$ sensitive optical probe, $DiOC_6/3/$, we examined the effect of β-lactam antibiotics on $\Delta\Psi$ of *S. griseus.* In this study we report our data about the depolarising effect of β-lactam antibiotics on $\Delta\Psi$ of *S. griseus* in relation to the β-lactam-induced cell autolysis of this strain.

MATERAL AND METHODS

Bacterial strains and culture conditions

The wild type streptomycin producer strain *Streptomyces griseus* NRRL B-2682 was isolated from the soil. Strains *spo1*$^-$, *spo2*$^-$, *spo3*$^-$ were newly isolated nonsporulating but A-factor responsive spontaneous mutants of *S. griseus* NRRL B-2682. Liquid media (100ml) were inoculated with 300 µl spore stock suspension and cultures were incubated in 500 ml Erlenmeyer flasks at 27 ^oC in a gyratory shaker at 250 r.p.m.. Solid cultures were grown on DM-agar (DM containing 2 % (w/v) Bacto agar), 150 µl spore stock solution was spread on the agar surface, than incubated at 27 ^oC. Spores of *spo-1* strain were produced by addition of

A-factor at 3×10^{-7} M concentration to DM-agar medium and this spore stock suspension was used to inoculate *spo-1* cultures.

Measurement of β-lactamase activity

The β-lactamase activity was determined by measuring the hydrolysis of nitrocefin (NC) at 37 ^{o}C for 10 min in 0.05 M KH_2PO_4 buffer (pH 7.0) in 1 ml as final volume. One unit was the amount of the enzyme that hydrolysis 1 μmol of NC / min at pH 7.0 and 37 ^{o}C.

Purification of β-lactamase of *Streptomyces griseus*

The extracellular β-lactamase present in the supernatant of 48-hour old cultures grown in E9 medium was precipitated with 70% (w/v) $(NH_4)_2SO_4$. The precipitate was resuspended in 0.075 M Tris-HCl buffer (pH 9.4) and desalted on Bio-Gel P6 gel-filtration column. Active fractions were collected and applied onto an FPLC chromatofocusing column in the same buffer. After chromatofocusing fractions containing high β-lactamase activity were collected and concentrated by ultrafiltration, and these samples were loaded onto a FPLC gel-filtration column using 0.05 M Tris-HCl (pH 7.0) as the eluent buffer.

The cellular membrane fractions were prepared as described by Barabás. Mycelium grown in GC medium was harvested by centrifugation. Cells were washed twice with 10 mM Tris-HCl buffer (pH 7.5) than resuspended in the same buffer containing 1 mg lysozyme ml^{-1} (Sigma) and 25 % (w/v) sucrose (Merck). Cells were incubated at 4 ^{o}C for overnight. Protoplasts were collected by centrifugation at 20000 g for 30 min at 4 ^{o}C. The pellet was resuspended in 0.05 M Tris-HCl (pH 7.2) buffer containing 5 mM $MgCl_2$, washed twice in the same buffer than centrifuged at 1100 g for 12 min at 4 ^{o}C. Cellular membrane fractions were precipitated by centrifugation at 20000 g for 30 min at 4 ^{o}C. The membrane bound form of the enzyme was released from the membrane by limited trypsine digestion at 37 ^{o}C for 30 min as described by Kharroubi and purified using the method described above. After gel-filtration the active fractions were precipitated with ice cold 10% (v/v) trichloroacetic acid (TCA) containing 125 μg deoxycholate ml^{-1}, precipitated samples were resuspended in sample buffer, heated at 80 ^{o}C for 5 min then loaded onto 13% SDS-polyacrylamide slab gel. After electrophoresis proteins from the gels were electrophoretically blotted to ProBlott™ immobilization membrane in a semi-dry system. N-terminal sequence analysis based on Edman degradation.

Preparation of protoplasts

Protoplasts were prepared with the method of Okanashi with slight modification. *S. griseus* NRRL B-2682 strain was grown in DM medium until the mid-exponential phase, then the mycelium was harvested by centrifugation, the pellet was washed twice in 0.1 M Tris-HCl buffer, recentrifugated as described above and suspended in the P-medium containing 0.1 mmol glucose and 1 mg/ml lysozime. The mixture was incubated at 27 ^{o}C for an hour. The protoplasts suspension was filtered through sterile cotton wool and washed with lysozime-free fresh P-medium. The protoplasts were collected by centrifugation and resuspended in P-medium. The concentration of protoplast samples was set to about $2x10^{8}$ protoplasts / ml medium.

Assay of the autolytic activity in the mycelium

The 15 hours old exponentially growing culture of *S. griseus* NRRL B-2682 was supplemented with N-acetyl-D-(1-^{3}H)glucosamine. After 3 hours of incubation the labelled mycelium was harvested by centrifugation, the pellet was washed twice with 0.1 M Tris-HCl and resuspended in fresh non-labelled DM medium. Aliquots of this suspension were withdrawn and treated with the tested reagents at zero time of incubation. The mycelial cultures were incubated at 27 ^{o}C. From time to time 200 µl aliquots were withdrawn from the suspension and centrifuged. From the supernatants 100 µl portions were taken off and the released radioactivity was determined by a liquid scintillation counter. The measured values were normalized to 1 mg of wet weight of the mycelium in the original culture.

Fluorescence measurements

All fluorescence measurements were carried out in a Hitachi Perkin-Elmer MPF-4 spectrofluorimeter equipped with a thermostated cell holder at 29 ^{o}C. Typically, the carbocyanine dye, $DiOC_6$/3/, was injected to 2.5 ml aliquots of the P-medium. The final dye concentration was set to 100 nM in all experiments. Protoplasts were added to the dye solution with gentle stirring, the final concentration was about 1-2 x 10^{-7} protoplasts / ml medium. After addition of protoplasts the system was allowed to attain equilibrium, reflected by the steady fluorescence of the dye, then the intensity was continuously recorded after injection of the test reagents (ionophores, antibiotics and salts). The fluorescence signals were evaluated on the basis of the response mechanism proposed by Sims for carbocyanine derivatives. The changes in the net fluorescence observed upon addition of test reagents were considered as changes in the partitioning of the dye between intra-and extracellular

compartments as well as the plasma membrane. Thus decrease of the fluorescence intensity (from the equilibrium value) upon addition of reagents was assumed to reflect hyprpolarization while an increase of the emission intensity was attributed to depolarisation of plasma membrane. These changes were checked and scaled with valinomycin and gramicidin ionophores as described earlier by several authors. The viability of the protoplasts was also controlled with gramicidin at the and of all measurements.

RESULTS

Time course of β-lactamase production during the life cycle of *S. griseus* NRRL B-2682 and its non-sporulating spontaneous mutant *spo1*$^-$ strain

1. In *S. griseus* NRRL B-2682 cells grown in liquid DM medium β-lactamase activity was first detectable in the cellular membrane of 5-hour-old cells, the extracellular form of the enzyme appeared much later, only in the supernatant of 24 h cultures. In contrast to this in the *spo1*$^-$ strain only the membrane bound form of the β-lactamase was present, its activity became detectable at the same age like that in the wild type strain.
2. This result was confirmed in two additional nonsporulating mutants, in the *S. griseus spo2*$^-$ and *spo3*$^-$ strains. None of them secreted extracellular β-lactamase activity, only the membrane-bound form of the enzyme was detectable in these strains during their whole life cycle.
3. Addition of AF to 6 h old of *S. griseus spo1*$^-$ cells grown in liquid DM medium restored not only the sporulation of the mutant as it was expected, but it also induced the secretion of the extracellular β-lactamase, even the time course of its secretion was the same as that of in the wild type strain.
4. When sporulation of *S. griseus* NRRL-B 2682 cells grown in liquid medium was inhibited by *m*-aminophenylboronic acid (*m*-APBA) or 0.5 % (w/v) casein hydrolysate, the extracellular β-lactamase activity was not detectable in the supernatant of cultures.
5. Globomycin, added to the DM liquid medium at 5 hours after inoculation, enhanced the secretion of the soluble form of the enzyme compared to that of the nontreated control cultures while parallel reduced the amount of the membrane-bound form. The total amount of the enzyme activity was not affected by globomycin at all. In the mutant strain the time course of the globomycin induced β-lactamase secretion was identical to that of *S. griseus* NRRL B-2682 cultures

6. When *S. griseus* NRRL B-2682 was grown in DM liquid medium containing 0.5 % (w/v) CH, where sporulation and extracellular β-lactamase production is suppressed, addition of the purified and digested cell wall fragments at 0.2 mg ml^{-1}, Ac_2-L-Lys-D-Ala-D-Ala at 0.5 mM and penicillin-G at 35 nM concentration restored the secretion of β-lactamase into the extracellular fluid, at the same time these compounds reduced the amount of membrane-bound form of the enzyme. The total β-lactamase activity remained the same in all cases.

N-terminal sequencing of extracellular β-lactamase of *S.griseus* NRRL B-2682

1. The extracellular and membrane-bound form of the β-lactamase from *S. griseus* NRRL B-2682 and membrane-bound form of the enzyme from the *spo1*$^-$ strain were purified to near homogeneity. After SDS-PAGE the relative molecular mass of the extracellular enzyme was found to be 31000 Da and its N-terminal sequence determined after electro blotting was the following: AAAPDIPIANVNA.

Characterization of the membrane-bound and the extracellular form of β-lactamase produced by *S. griseus* NRRL B-2682 and its nonsporulating mutant.

1. Among the substrates tested, benzylpenicillin was the best substrate for the various forms of the β-lactamase, ampicillin was hydrolysed at a relatively similar rate in all cases. The first generation type cefamandole and cefaloridine were poor substrates for all forms, while other cephalosporines, like cefuroxime, cefotaxime and ceftazidine were not hydrolysed at all.
2. The effect of various kinds of inhibitors on the β-lactamase activity was determined, 1 mM *m*-APBA showed the strongest inhibitory effect on the enzyme activity and 1 mM *p*-chloromercuribenzoate (pCMB) significantly inhibited the β-lactamase activity too.
3. The K_i values of some of the well-known β-lactamase inhibitors were also determined. Both the extracellular and membrane-bound form of the β-lactamase of *S. griseus* NRRL B-2682 and the membrane-bound enzyme of the *spo1*$^-$ mutant were susceptible to inhibition by clavulanic acid and sulbactam, while cloxacillin had a weaker affinity towards them.

Effect of penicillin G, purified and autolysins digested cell wall fragments, Ac_2-L-Lys-D-Ala-D-Ala and adjuvant peptide (N-AcMur-L-Ala-D-isoglutamine) on the growth and differentiation of *S. griseus* NRRL B-2682

Study of cultures grown on solid media

Effect of β-lactam antibiotics

1. Using the gradient-plate method we have shown, that *S. griseus* spores, inoculated on DM medium containing penicillin G antibiotic gradient and the sporulation inhibitor 0.5 % CA, formed aerial mycelium after 36 h of incubation. In addition, some mature spore formation was observed. Using the agar-diffusion method we have proved the age dependent effect of penicillin G on spore formation. Adding 34 nmol penicillin G to 24 hours old cultures showed no effect neither on aerial mycelium formation nor on spore formation.

2. Using the agar-diffusion test, we have studied the effect of the β-lactam antibiotics. Similarly to penicillin, 25 nmol ampicillin, 24 nmol cephaloridine and 28 nmol cephamandole showed the same age-dependent effect on differentiation.

3. Using the previously mentioned agar-diffusion method under similar conditions, we tested the effect of 6-APA, the inactive precursor of penicillin. 6-APA was ineffective in all the experiments.

Effect of cell wall fragments

1. Agar diffusion method was used to study the effect of cell wall peptides and NAM on differentiation under the previously mentioned conditions. All tested molecules (0.2 mol D-Ala-D-Ala, 0.2 μmol Ac_2-L-Lys-D-Ala-D-Ala, 0.4 μmol NAM) restored the normal differentiation of *S. griseus*.

2. In identical control experiments we have used amino acids and peptides that are not component of the peptidoglycan and in those cases no effect on the differentiation process of CH suppressed *S. griseus* NRRL B-2682 cells was observed.

Effect of inhibitors of peptidoglycan synthesis

1. 3×10^{-4} μmol vancomycin, one of the cell wall biosynthesis inhibitor, restored aerial mycelium formation in an age- and concentration dependent fashion, under sporulation repression. After 36 hours of incubation spore formation was observed.

2. Interestingly, antibiotics (bacitracin, fosfonomycine, cycloserine) inhibiting previous steps of cell wall synthesis did not show any effect on differentiation.

Examination of cultures grown on solid media

1. S. griseus NRRL B-2682 is able to sporulate in liquid cultures. Penicillin-G at subinhibitory concentration (33 nmol) counteracted the inhibitory effect of CH on the normal differentiation and restored spore formation.

2. In similar experiments we have found that addition of 0.3 mg cell wall precursors, 0.2 μmol Ac_2-L-Lys-D-Ala-D-Ala or 0.25 μmol adjuvant peptide to *S. griseus* NRRL B-2682 cultures grown in the presence of 0.5 % CH also resulted in mature spore production too When the same amount from these agents were added to 20 h old cells of *S. griseus* NRRL B-2682 these agents were ineffective.

3. In identical control experiments we have used amino acids and peptides that are not component of the peptidoglycan and in those cases no effect on the differentiation process of CH suppressed *S. griseus* NRRL B-2682 cells was observed.

The effect of the ionophore gramicidin D and penicillin G on the cell wall lysis of *S. griseus* NRRL B-2682

1. In the second part of our experiments we studied whether a change in ΔΨ of cytoplasmic membrane has an effect on cell wall autolysis. We studied the effect of penicillin G and gramicidin D effect on the release of N-acetyl-D-(1-^{3}H)glucosamine labelled cell wall fragments in *S. griseus*.

2. Addition of gramicidin D and penicillin G caused a concentration-dependent rise in the amount of released, labelled cell wall material compared to that of the untreated culture. The penicillin G induced cell wall lysis started earlier and the difference between the penicillin G and gramicidin D treated samples was especially prominent during the first 5 h of the measurement.

The effect of the ionophore gramicidin D and penicillin G on the membrane potential (ΔΨ) in *S. griseus* NRRL B-2682

1. In parallel experiments the effect of gramicidin D and penicillin G on ΔΨ was also investigated by means of a ΔΨ sensitive fluorescent dye, the $DiOC_6(3)$. To study this effect of gramicidin D and penicillin G we choose to use protoplasts prepared from early-exponentially growing cells.

2. Our results demonstrated that gramicidin D depolarised ΔΨ as expected and secondary the change in the ion gradient through the cytoplasm membrane resulted in increased cell wall lysis in *S. griseus.*

3. Addition of 2 × MIC of penicillin G to protoplasts induced a stepwise, increasing but partial depolarisation of ΔΨ. Even after three successively added doses of penicillin G it was still possible to further reduce ΔΨ by adding gramicidin D.

4. The depolarising effect of penicillin G is concentration dependent. Addition of 5 × MIC of penicillin G induced a larger depolarisation in ΔΨ than 2 × MIC, and a second dose of 5 × MIC of penicillin G almost completely depolarised the ΔΨ.

5. The depolarising effect of penicillin G showed age dependence too. When 5 × MIC of penicillin G was added to older protoplasts a single dose had no effect at all.

6. Since β-lactamase activity is already detectable in the membrane of *S. griseus* cells right after germination it is possible that not penicillin G itself, but its cleaved, inactivated form, the penicilloic acid has the detected depolarisation effect on ΔΨ. However, testing the effect of penicilloic acid showed that penicilloic acid even at the concentration of 10^{-3} M did not depolarise ΔΨ of *S. griseus* protoplasts.

7. Ampicillin and cephalosporin antibiotics depolarised ΔΨ of the protoplasts in age and concentration dependent manner, although none of the β-lactam compounds were as good of a depolarising agent as penicillin G. After ampicillin and cephalosporin treatment, gramicidin D still effectively depolarised ΔΨ of protoplast membranes.

DISCUSSION

Our results revealed that *S. griseus* NRRL B-2682 produced a membrane-bound and an extracellular form of a β-lactamase, while in its non-sporulating spontaneous mutant *spo-1* strain only the membrane-bound enzyme activity was found. Since all of the β-lactamases isolated so far from *Streptomyces* spp. have been reported to be constitutively synthesized extracellular enzymes, to our knowledge this is the first example of a membrane-bound β-lactamase isolated from *Streptomyces.*

Based on our results we concluded that β-lactamase enzyme remains bound to the cytoplasm membrane when spore formation was inhibited (0.5 % CH or 15 μmol APBA) in the culture. When non-sporulating mutant species (*spo1*$^-$*, spo2*$^-$*, spo3*$^-$) were forced to sporulate with AF, the mutants parallel with the appearance of spores excrete the enzyme into the excracellular space. These results support our hypothesis that in *S. griseus* β-lactamase enzyme secretion coupled with its differentiation process.

In our experiments, globomycin - which specifically inhibits the bacterial signal peptidase II responsible for the processing of the membrane-bound lipoproteins only - prevented the accumulation of the membrane-bound β-lactamase enzyme both in the wild type and mutant strain. We assume that in our system globomycin inhibited the processing of the prelipoprotein form of the enzyme, which resulted in the inhibition of the formation of the fatty acid-amide linkage on the N-terminal Cys residue of the protein. As a consequence the imprecisely processed protein, which had only the glyceride-thioether modification, was not retained in the membrane and accumulated in the extarcellular space. Our results indicate that in *S. griseus* NRRL B-2682 β-lactamase is synthesized first as a prelipoprotein and after proper processing the enzyme has a posttranslational modified membrane-bound form which is later released to the extracellular space.

The extracellular and membrane-bound form of the β-lactamase from *S. griseus* NRRL B-2682 and membrane-bound form of the enzyme from the *spo-1* strain were purified to near homogeneity. After SDS-PAGE the relative molecular mass of the extracellular enzyme was found to be 31000 Da and its N-terminal sequence determined after electro blotting was the following: AAAPDIPIANVNA. Comparison of the N-terminal sequence of *S. griseus* NRRL B-2682 β-lactamase with sequences of the FastA databank showed 81.8 % homology between the β-lactamase and the N-terminal sequence -APDIPLANVKA- of the D-aminopeptidase enzyme of another *S. griseus* strain. It is not surprising, since based on similar characteristics of β-lactamase enzyme, D-aminopeptidase enzyme and PBP, and because they catalyse similar reactions, these enzymes belong to the penicillin-recognizing superfamily.

All forms of the enzyme showed much higher activity (as reflected by their higher V_{max} values) towards penicillin than against cephalosporins and the strong inhibitory effect of clavulanic acid on β-lactamase activity also suggested that these enzymes behave as penicillinases. The inhibitory effect of *m*-APBA indicated the presence of active-site serine residue(s) in both forms of the enzyme, as it was found in the case of other *Streptomyces* penicillinases, which are generally serine β-lactamases. Inhibition of β-lactamase activity by pCMB suggests the presence of catalytically important cystein residue(s) in the enzyme. According to these results we suggest that the membrane-bound and extracellular forms of the enzyme are closely related to each other (probably they are differently processed forms of the same protein) and they seem to belong to class A β-lactamases.

We have studied a possible connection between cell wall biosynthesis and β-lactamase production, so we have analysed the effect of purified and autolysin digested cell wall

fragments, the cell wall precursor Ac_2-L-Lys-D-Ala-D-Ala and penicillin G on the β-lactamase production of *S. griseus* NRRL-B2682. Our results indicated that in cultures of the wild type strain the secretion of β-lactamase into the extracellular space was enhanced by addition of these agents to cells grown in 0.5 % CH supplemented DM liquid medium (under sporulation repressed condition). However, no induction of the β-lactamase gene was observed, indicating that the enzyme is constitutive in *S. griseus* NRRL-B 2682. Thus, it can be concluded that the increased level of cell wall fragments/precursors are important factors to affect the release of the β-lactamase from the cellular membrane. So we propose that there might be a link between β-lactamase secretion and the turn-over of cell wall fragments in *S. griseus* NRRL-B2682.

While studying the effect of cell wall fragments, precursors and β-lactam antibiotics on β-lactamase secretion we have noticed that these agents produced significant morphological changes in sporulation repressed cultures of wild type *S. griseus*. They suspended the inhibitory effect of CH and restored the ability of cells to produce spores on solid and in liquid media in an age-dependent manner. All of our results suggest that the increased amount of cell wall precursors both in the extra- and intracellular space influences the differentiation process of *S. griseus* 2682, they might serve as a signal for the initiation of sporulation process. The restoration of the normal differentiation process was invariably coupled to the secretion of the extracellular β-lactamase. Probably the bacterium uses the peptidoglycan turn-over as a general signalling mechanism to obtain information about the peptidoglycan, and to able to react to those changes which may threaten the mechanical stability of peptidoglycan. This assumption is strengthened by the fact, that 6-APA and non-cell wall component amino acids had absolutely no effect on differentiation.

We have also found it very interesting, that from other tested cell wall inhibitor antibiotics (bacitracin, vancomycine, cycloserine, phosphonomycine), only vancomycin restored aerial mycelium and spore formation in an age- and concentration dependent fashion.

We suggest that there may be a connection between the cell wall synthesis and the differentiation process of *S. griseus* 2682 and there must be a system that is able to sense the state of the cell wall biosynthesis and mediate this signal to the genetic apparatus of the strain. In the future we would like to clarify whether β-lactamase could take part in this signal sensing/transduction process, whether β-lactamase may play a role in monitoring the status of cell wall synthesis.

We have studied induction of autolysis in *S. griseus*, so the murein sacculus of cells in the early-exponential growth phase was labelled with N-acetyl-D-(1-^{3}H)glucosamine. Incubation of labelled cells in the presence of gramicidin D or penicillin G resulted in a concentration-dependent increase in the release of labelled cell wall material. Using the ΔΨ sensitive fluorescent dye $DiOC_6$3 we have demonstrated that gramicidin D immediately depolarised ΔΨ of *S. griseus* cells. These results suggest that the gramicidin D induced depolarisation of ΔΨ indeed triggers cell wall lysis in this bacterium. Using the same fluorescence method we have found that penicillin G immediately depolarised ΔΨ of *S. griseus* protoplasts in a concentration and age dependent manner. Older protoplasts were not effected even by higher amount of penicillin G. The resistance toward the depolarising effect of penicillin G in older protoplasts was not due to the presence of β-lactamase activity in the membrane of protoplasts, since pre-treatment of protoplasts with β-lactamase inhibitor clavulanic acid did not augment the affectivity of penicillin G. Penicilloic acid, the hydrolysed biologically inactive derivate of penicillin G did not alter the ΔΨ of protoplasts. This age-dependent effect of penicillin G on ΔΨ correlates well with its *in vivo* growth stage dependent bacteriolytic effect.

The same result was found when the effect of ampicillin and cephalosporin antibiotics were analysed in similar assay, all tested agents induced a dose and age dependent depolarisation of ΔΨ.

Together these findings support the hypothesis that the electrophysiological state of the cellular membrane is an important factor in the regulation of cell wall autolysis. β-lactam antibiotics depolarise ΔΨ of *S. griseus* cells and the drop in ΔΨ could be a signal to trigger autolysis. Depolarisation of ΔΨ alone, however, is not enough to induce a massive autolysis. It is possible, that the combined effect of the inhibition of peptidoglycan biosynthesis and depolarisation of ΔΨ together make β-lactams so powerful antibiotics. We would like to emphasise that depolarising effect of β-lactam molecules is not a result of the inhibition of murein cell wall biosynthesis, instead a direct effect, because protoplasts do not have cell walls. However, the molecular mechanism of β-lactam antibiotic provoked depolarisation is not completely understood. We do not know, whether PBP-s and other target molecules, like holins, play a role in this mechanism. In addition it is not clear either, how the decrease in the membrane potential leads to the induction of the cell wall lysis. Based on these an interesting hypothesis can be proposed: penicillin provoked depolarisation resulting in holing-like protein activation and the inhibition of cell wall biosynthesis leads to massive bacterial cell wall lysis.

Our experimental findings are also in agreement with results of recently published flow cytometric measurements, which showed that ampicillin treatment of *Staphylococcus aureus, S. luteus* and *Micrococcus luteus* cells resulted in ΔΨ depolarisation.

Based on our findings, we assume, that β-lactam antibiotic induced depolarisation of ΔΨ in *S. griseus* plays an important role in triggering cell wall autolysis, which is a well known result of β-lactam treatment.

SUMMARY

A new type of β-lactamase has been isolated from *S. griseus* NRRL B-2682, which has membrane-bound and extracellular form. Comparison of the characteristics of the membrane-bound and extracellular enzymes suggests that they seem to be differently processed forms of the same enzyme. According to the biochemical characterisation the β-lactamase of *S. griseus* belongs to the class A of penicillinases. The N-terminal amino acid sequence of the extracellular enzyme showed a high degree of similarity to a D-aminopeptidase of another *S. griseus* strain.

Secretion of the β-lactamase was affected by the differentiation state of the strain since in its non-sporulating mutants only the membrane-bound form was present. In accordance with this, when sporulation of the wild type strain was inhibited it failed to secrete the enzyme into the extracellular space. Addition of globomycin to the non-sporulating cells liberated the enzyme from the membrane, indicating, that the protein is processed normally by signal-peptidase II and a glyceride-thioether group, together with the fatty acid amide-linkage, is responsible for the attachment of the enzyme to the cellular membrane.

Under sporulation-repressed conditions addition of peptidoglycan fragments and analogues or inhibition of cell wall biosynthesis by β-lactam antibiotics (penicillin, ampicillin or different type of cephalosporins) or vancomycin induced β-lactamase secretion and also restored sporulation both in solid and submerged cultures. These results confirm that β-lactamase secretion is tightly coupled to the sporulation process in *S. griseus*.

The effects of β-lactam antibiotics, inhibitors of cell wall biosynthesis and inducers of cell wall autolysis, were studied on plasma membrane electrophysiology in *S. griseus* NRRL B-2682. Using the $DiOC_6 3$ fluorescents ΔΨ sensitive assay, we examined the effect of β-lactams on the membrane potential of *S. griseus* protoplasts. We have shown that addition of various β-lactam antibiotics induced a dose- and growth stage dependent depolarisation of the

membrane potential of *S. griseus*. Inactive derivates of β-lactam compounds, like 6-APA, penicillin amine and the hydrolysed biologically inactive penicilloic acid had no depolarising effect on the membrane potential. The ionophore gramicidin D, while depolarising the membrane potential, also induced a dose dependent increase in cell wall lysis. These observations suggest that alteration of the transmembrane potential could be an important signal in triggering cell wall autolysis of *S. griseus*.

List of publication

The dissertation is based on the following publications:

Penyige, A., **Deák**, **E.,** Kálmánczhelyi, A. and György Barabás: Evidence of a role for NAD^+-glycohydrolase and ADP-ribosyltransferase in growth and differentiation of *Streptomyces griseus* NRRL B-2682: inhibition by aminophanylboronic acid. (1996).
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Deák, E., Szabó, I., Kálmánczhelyi, A., Gál, Zs., Barabás, Gy. and András Penyige: Membrane-bound and extracellular β–lactamase production with developmental regulation in *Streptomyces griseus* NRRL B-2682. (1998).
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