Membrane-bound and extracellular β -lactamase production with developmental regulation in *Streptomyces griseus* NRRL B-2682

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A new type of β -lactamase has been isolated and characterized in *Streptomyces griseus* NRRL B-2682. The enzyme has membrane-bound and extracellular forms. Biochemical characterization of some of the properties of the enzyme showed that it belongs to the class A group of penicillinases. Comparison of the membrane-bound and extracellular forms of the β -lactamases suggests that they seem to be differently processed forms of the same enzyme. The N-terminal amino acid sequence of the extracellular form of the β -lactamase showed a high degree of similarity to a D-aminopeptidase of another *Streptomyces griseus* strain. Secretion of the β -lactamase was affected by the differentiation state of the strain since in spontaneous 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 extracellular β -lactamase. 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 a 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 penicillin-G 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*.

Keywords: *Streptomyces griseus*, β -lactamase, sporulation

INTRODUCTION

Streptomyces spp., representatives of the actinomycetes, are soil bacteria known for their elaborate life cycle. 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. This cell type will form the mature spores by a

synchronous septation process. *S. griseus* also sporulates abundantly in liquid culture, a rare ability not characteristic of most *Streptomyces* strains (Kendrick & Ensign, 1983). Therefore, *S. griseus* provides an ideal model to study prokaryotic development.

In several *Streptomyces* strains morphological differentiation, antibiotic production and resistance depend on the presence of small diffusible extracellular auto-regulatory factors (Barabás *et al.*, 1994). One of the best known molecules among them is A-factor (AF) (γ -2S-isocaprolyl-3S-hydroxymethyl- γ -butyrolactone) (Hori-nuchi & Beppu, 1992; Khoklov *et al.*, 1973; Miyake *et al.*, 1990). *S. griseus* mutants which do not produce this autoregulator are typically non-sporulating bald mutants. However, both antibiotic production and

Abbreviations: AF, A-factor; CA, Casamino acids; GS, glutamine synthetase; *m*-APBA, *m*-aminophenylboronic acid.

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sporulation can be restored by the addition of exogenous AF.

Streptomyces are also known to produce a wide variety of extracellular enzymes, including β -lactamases (Ogawara, 1981). Certain *Streptomyces* strains produce β -lactam compounds, some of which have antibacterial activity and some which act as β -lactamase inhibitors. One possible explanation for the emergence of β -lactamases in Gram-positive antibiotic-producing bacteria is that they produce these enzymes to protect themselves from their own secondary metabolites. In producer strains the self resistance genes are part of the antibiotic biosynthetic gene cluster (Pérez-Llarena *et al.*, 1997; J. Martin, personal communication). Approximately half of *Streptomyces* strains, however, produce β -lactamases without any apparent relationship to self resistance to β -lactam compounds (Ogawara, 1996), since most of them are β -lactam non-producers (Paradkar *et al.*, 1996).

In several Gram-positive bacteria β -lactamase expression can be induced by the addition of penicillin or peptidoglycan precursors to the medium, as shown in *Bacillus licheniformis* (Zhu *et al.*, 1992; Joris *et al.*, 1994), but in most *Streptomyces* strains β -lactamase production is not inducible (Ogawara, 1981). Rare exceptions are the well known β -lactamases found in *Streptomyces cacaoi* (Lenzini *et al.*, 1992; Juana *et al.*, 1992; J. Dusart personal communication). In *Escherichia coli* induction requires the presence of the AmpR protein and the transport of mucopeptides into the cytoplasm (Jacobs *et al.*, 1994, 1997). In *Enterobacter cloacae* changes in the structure and composition of the cell wall peptidoglycan, caused by the addition of glycine and D-amino acids to cultures, resulted in β -lactamase induction (Ottolenghi *et al.*, 1993).

In most cases 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 of Gram-negative bacteria (Nielsen & Lampen, 1982; Oudega *et al.*, 1993). It is also known, however, that in *B. licheniformis* and in *Bacillus cereus* 569/H the Class A β -lactamase is present both as a membrane-bound hydrophobic form as well as a soluble exoenzyme (Nielsen & Lampen, 1982, 1983; Hussain *et al.*, 1987).

Membrane-bound lipoproteins in bacteria are synthesized as prelipoprotein precursors containing N-terminal signal peptides which are subsequently modified by addition of lipids (Wu, 1985; Lampen *et al.*, 1986; Hayashi & Wu, 1990). The lipophilic modification consists of a diacyl-glycerol group forming a thioether linkage with an N-terminal cysteine residue and a third long-chain fatty acid attached to the same cysteine through an amide linkage (Nielsen & Lampen, 1983). The N-terminal extension carrying the lipophilic modification is thought to be responsible for membrane anchorage. In later stages of the life cycle these lipoproteins might be excreted into the extracellular space by further processing of the enzyme (Lampen *et al.*,

1986; Nagarajan, 1993). Globomycin is a cyclic peptide antibiotic which is known to inhibit the processing of bacterial prelipoproteins (Inukai *et al.*, 1978). This inhibition results in secretion of the enzymes into the extracellular space in Gram-positive bacteria (Lampen *et al.*, 1986).

In this publication we show that *S. griseus* NRRL B-2682 produces a membrane-bound and an extracellular form of a β -lactamase and the release of this enzyme correlates with the differentiation state of the strain. This is further supported by the finding that in a spontaneous, non-sporulating mutant of the wild-type strain, *S. griseus spo-1*, only the membrane-bound form of β -lactamase could be detected.

METHODS

Bacterial strains and culture conditions. The wild-type streptomycin producer strain *Streptomyces griseus* NRRL B-2682 was a generous gift from J. C. Ensign, Department of Bacteriology, University of Wisconsin-Madison, USA. Strains *spo-1*, *spo-2* and *spo-3*, which are spontaneous non-sporulating AF-responsive mutants of *S. griseus* NRRL B-2682, were isolated in our laboratory. Cells were grown in the following media. (i) Defined medium (DM) containing 25 mM Tris/HCl (pH 7.2), 50 mM glucose, 5 mM NH_4Cl , 0.5 mM MgCl_2 , 10 mM KH_2PO_4 , 0.05% Casamino acids (CA) (Serva) and 0.4% trace salt (TS) solution (TS is 17 mM CaCl_2 , 5 mM FeSO_4 , 20 mM MnSO_4 , 0.3 mM ZnSO_4); in some experiments DM was supplemented with 0.5% CA to inhibit the ability of the cells to produce aerial mycelia and spores). (ii) E9 medium containing 10 mM CaCO_3 , 47 mM NaNO_3 , 12 mM K_2HPO_4 , 6 mM KCl, 2 mM MgSO_4 , 50 mM glucose, 0.5% Bacto peptone and 0.5% yeast extract (pH 7.3). (iii) Glycerin-casein (GC) medium containing 86 mM NaCl, 10 mM CaCO_3 , 4 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 57 mM K_2HPO_4 , 3 mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 35 mM KOH and 0.05% CA (pH 7.6). Liquid media were inoculated with spore suspension and cultures were incubated at 27 °C on a gyratory shaker at 250 r.p.m. Solid cultures were grown on DM-agar [DM containing 2% (w/v) Bacto agar], spore solution was spread on the agar surface and then incubated at 27 °C. Spores of the *spo-1* strain were produced by addition of AF at 3×10^{-7} M to DM-agar and this spore suspension was used to inoculate *spo-1* cultures.

Purification of β -lactamase of *Streptomyces griseus* NRRL B-2682. The extracellular β -lactamase present in the supernatant of 48-h-old cultures grown in E9 medium was precipitated with 70% (w/v) $(\text{NH}_4)_2\text{SO}_4$. The precipitate was resuspended in 0.075 M Tris/HCl buffer (pH 9.4) and desalted on a Bio-Gel P6 gel-filtration column (10 $\times$ 100 mm, Bio-Rad). Active fractions were collected and applied onto an FPLC chromatofocusing column (MonoP HR 5 $\times$ 200 mm, Pharmacia) in the same buffer. The enzyme was eluted with a pH gradient from 9.4 to 6.0 using tenfold-diluted Polybuffer 96 (Pharmacia) at a flow rate of 1 ml min⁻¹. Fractions containing high β -lactamase activity were collected and concentrated by ultrafiltration (Millipore NMWL 10000 filter). The concentrated samples were loaded onto an FPLC gel-filtration column (0.8 $\times$ 300 mm, Waters SW 200) using 0.05 M Tris/HCl (pH 7.0) as the eluant buffer at a flow rate 1 ml min⁻¹ and active fractions were collected and concentrated as before.

The cellular membrane fractions were prepared as described by Barabás *et al.* (1988). Mycelium grown in GC medium was harvested by centrifugation at 4000 g for 25 min at 4 °C. Cells

were washed twice with 10 mM Tris/HCl buffer (pH 7.5) then resuspended in the same buffer containing 1 mg lysozyme ml⁻¹ (Sigma) and 25 % (w/v) sucrose (Merck). Cells were incubated at 4 °C overnight and the protoplasts were collected by centrifugation at 2000 g for 5 min at 4 °C. The pellet was resuspended in 0.05 M Tris/HCl buffer (pH 7.2) containing 5 mM MgCl₂, washed twice in the same buffer and centrifuged at 1100 g for 12 min at 4 °C. Cellular membrane fractions were pelleted by centrifugation at 20000 g for 30 min at 4 °C. The membrane-bound form of the enzyme was released from the membrane by limited trypsin digestion at 37 °C for 30 min as described by Kharroubi *et al.* (1989) and purified using the method described above. After gel filtration the active fractions were precipitated with ice-cold 10 % (v/v) TCA containing 125 µg deoxycholate ml⁻¹. Precipitated samples were resuspended in sample buffer (Laemmli, 1970), heated at 80 °C for 5 min and then loaded onto 13 % SDS-polyacrylamide slab gels. SDS-PAGE was carried out with 35 mA constant current at 4 °C. The molecular mass of the enzyme was determined by using an SDS-7 (Sigma) molecular mass standard mixture. After electrophoresis gels were equilibrated in 25 mM Tris/HCl (pH 9.4) containing 10 % (v/v) methanol and 192 mM glycine (cathode buffer), and subsequently proteins from the gels were electrophoretically blotted to ProBlott™ (Applied Biosystems) immobilization membrane using a semi-dry system. The following buffers were used for the transfer: (i) Anode buffer I, 0.3 M Tris base containing 10 % methanol (pH 10.4); (ii) anode buffer II, 25 mM Tris base containing 10 % methanol (pH 9.4); and (iii) cathode buffer as described above. During the transfer a 2 mA cm² constant current was applied for 1 h and then the blots were stained with Coomassie brilliant blue.

N-terminal sequencing. Sequence analysis based on Edman degradation was performed by a model 910 Knauer Protein Sequencer. The N-terminal sequence of the protein was determined from samples electrotransferred to ProBlott membranes after SDS-PAGE.

Measurement of β -lactamase activity. (i) β -Lactamase activity was determined by measuring the hydrolysis of nitrocefin at 37 °C for 10 min in 0.05 M KH₂PO₄ buffer (pH 7.0) in 1 ml final volume. One unit was defined as the amount of enzyme that hydrolysed 1 µmol nitrocefin under these conditions (O'Callaghan *et al.*, 1972). (ii) Spectrophotometric measurements were carried out in a Shimadzu UV 3000 spectrophotometer equipped with a thermostat cell holder at 37 °C (Samuni, 1975). For the various substrates the enzyme activity was calculated using the corresponding $\Delta\epsilon(\lambda)$, where one unit of enzyme activity was defined as the amount of enzyme that hydrolysed 1 µmol substrate min⁻¹ at 37 °C, pH 7.0.

Preparation of crude cellular extract for glutamine synthetase (GS) assays. Crude extracts for GS assays were prepared from mycelia grown in DM medium. Mycelia were centrifuged at 6000 g for 20 min and then cells were disrupted by brief sonication with 30 s bursts for 2 min at 4 °C using a Branson Sonifier model 250. The disrupted cell suspension was centrifuged at 12000 g for 20 min at 4 °C and the resulting supernatant was used as crude cellular extract preparation. The same method was used to prepare cellular membrane fractions to detect membrane-bound penicillinase activity.

GS assay. GS activity was determined by using the γ -glutamyltransferase assay (Bender *et al.*, 1977). In control experiments reaction mixtures were also prepared without arsenate and ADP to subtract the amount of γ -glutamyl-

hydroxamate produced by enzymes other than GS (less than 3 % of total activity). One unit of enzyme activity was defined as the amount of protein that formed 1 mmol γ -glutamylhydroxamate min⁻¹ at 37 °C.

Preparation of cell wall fragments. *S. griseus* cells were disrupted by ultrasonic treatment and cell wall fragments were isolated by the differential centrifugation method (Szabó *et al.*, 1989). The purified cell wall, still containing autolytic enzymes(s), was subjected to exhaustive autolysis by incubating samples in 0.1 M ammonium formate buffer (pH 9.0) at 5 °C for 3 d, followed by lyophilization.

RESULTS

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

The extracellular and membrane-bound forms of the β -lactamase from *S. griseus* NRRL B-2682 and the membrane-bound form of the enzyme from the *spo-1* strain were purified to near homogeneity. During the chromatofocusing step the isoelectric point of both forms of the enzyme was pH 7.2 and their relative molecular masses were found to be 31000 Da on SDS-PAGE, according to their relative mobility (Fig. 1). The N-terminal sequence of the extracellular enzyme determined after electroblotting was AAAPDIPIANVNA. Comparison of the first 11 aa of this sequence with sequences present in the EBI database showed 81.8 % similarity to the N-terminal sequence, APDIPLANVKA,

Fig. 1. Coomassie-brilliant-blue-stained electroblot analysis of the purified extracellular and membrane-bound β -lactamase of *S. griseus* NRRL B-2682. Proteins were separated by SDS-PAGE (13 %) and then electrophoretically transferred from the gel to ProBlott membrane. Lanes represent samples from concomitant purification steps: 1, 2, active fractions after chromatofocusing of the precipitated extracellular proteins; 3, purified membrane-bound enzyme after gel filtration; 4, purified extracellular enzyme after gel filtration; 5, molecular mass markers. The relative molecular mass of the membrane-bound and the extracellular β -lactamase was found to be 31000 Da.

Fig. 2. Comparison of the time course of β -lactamase production in *S. griseus* NRRL B-2682 and in non-sporulating mutant strain *spo-1*. Cells were grown in DM liquid medium at 27 °C and β -lactamase activity was measured at the indicated time in the isolated cellular membrane (□, ■) and in the supernatant (△, ▲) of the wild-type and the *spo-1* mutant strain, respectively. Values are means of three independent measurements.

of the D-aminopeptidase enzyme of another *S. griseus* strain (Maras *et al.*, 1996). Our enzyme did not show aminopeptidase activity. Previously, another β -lactamase was also found to show similarity to D-aminopeptidases (Asano *et al.*, 1992).

Time course of β -lactamase production during the life cycle of *S. griseus* wild-type and *spo* mutants

To study β -lactamase production in the wild-type *S. griseus* NRRL B-2682 and in its spontaneous non-sporulating mutant, *spo-1*, the localization of β -lactamase and the time course of its expression were determined first. In wild-type cells grown in liquid DM medium, β -lactamase activity was initially detected in the cellular membrane of 5-h-old cells, while the extracellular form of the enzyme appeared much later at 24 h. In contrast to this, in the *spo-1* strain only the membrane-bound form of the β -lactamase was present; the time course of its activity profile was similar to the wild-type strain (Fig. 2). The appearance of a low level of extracellular β -lactamase activity in 40-h-old *spo-1* cultures was very likely due to lysis of a certain proportion of the cells. This result was confirmed in two additional non-sporulating mutants, *spo-2* and *spo-3*. Neither of these two strains secreted extracellular β -lactamase activity; only the membrane-bound form of the enzyme was detectable during their whole life cycle (data not shown). As shown in Table 1, addition of 80 μ g AF ml^{-1} at 6 h after inoculation to the *spo-1* strain grown in liquid DM medium induced the secretion of the extracellular β -lactamase with a time course profile

Table 1. Effect of AF on β -lactamase secretion of the *S. griseus spo-1* mutant

Cells were grown in DM liquid medium at 27 °C and sampled at 26, 32 and 48 h after inoculation. AF was added at 80 μ g ml^{-1} to 6-h-old cells and β -lactamase activity was measured in the supernatant of the culture after mycelium was harvested by centrifugation at 4000 g for 30 min at 4 °C.

Strain	β -Lactamase specific activity (units)		
	26 h	32 h	48 h
<i>S. griseus</i> NRRL B-2682	1×10^{-3}	3×10^{-3}	5×10^{-3}
<i>S. griseus spo-1</i>	0	0	1.2×10^{-4}
<i>S. griseus spo-1</i> + AF	0	1.3×10^{-3}	2.7×10^{-3}

similar to that of the wild-type strain and sporulation of the mutant was also restored as expected.

Previously we have reported that low concentrations (15 μ M) of *m*-aminophenylboronic acid (*m*-APBA) (Penyige *et al.*, 1996) or 0.5 % casein hydrolysate (de-los-Reyes-Gavilán *et al.*, 1991) inhibited sporulation of *S. griseus* NRRL B-2682 cells in liquid medium. When sporulation was suppressed by addition of these agents extracellular β -lactamase activity was not detected in the supernatant of wild-type cultures (data not shown).

Characterization of the membrane-bound and extracellular forms of β -lactamase in *S. griseus* wild-type and *spo-1* mutant

To characterize the enzyme we isolated both forms of the β -lactamase from wild-type cultures and the membrane-bound form from the *spo-1* mutant strain. Substrate specificity of the purified enzymes was determined against various β -lactam compounds, such as benzylpenicillin and ampicillin, as well as first, second and third generation cephalosporins as described by Bush (1989). Among the substrates tested, benzylpenicillin was the best substrate for the various forms of the β -lactamase. Ampicillin was hydrolysed at a similar rate in all cases. The first generation cephalosporins, cefamandole and cephaloridine, were poor substrates for all forms, while other cephalosporins, like cefuroxime, cefotaxime and ceftazidime were not hydrolysed at all. According to these results the β -lactamases we have isolated are penicillinases rather than cephalosporinases.

The effect of various kinds of inhibitors on the activity of purified β -lactamases was also determined using nitrocefin as substrate (Bush, 1989). Among the compounds tested *m*-APBA at a concentration above 1 mM showed the strongest inhibitory effect on the enzyme activity and 1 mM *p*-chloromercuribenzoate was also significantly inhibitory. The inhibitory effect of *p*-chloromercuribenzoate could be reversed by addition of equimolar amounts of cysteine (data not shown).

Fig. 3. Effect of globomycin on growth, β -lactamase production and secretion of *S. griseus* NRRL B-2682 (a) and non-sporulating mutant strain *spo-1* (b) grown in DM liquid medium. Globomycin was added at $50 \mu\text{g ml}^{-1}$ to 5-h-old cells. β -Lactamase activity was measured in the membrane fraction in the absence ($\square$) and presence (∇) of globomycin and in the extracellular space in the absence ($\triangle$) and presence ($\diamond$) of globomycin. Total penicillinase production ($\times$) was determined; this was not affected by globomycin (data not shown). The amount of protein ($\circ$) in the sample was determined by the Lowry method.

Inhibition by *m*-APBA may suggest the presence of active site serine residue(s) in the enzyme, while the inhibitory effect of *p*-chloromercuribenzoate predicts the presence of a catalytically important cysteine residue in these β -lactamases (Bush, 1989). NaCl (100 mM) and (1 mM EDTA) did not affect β -lactamase activity.

The K_i values of some of the well-known β -lactamase inhibitors were also determined (Bush, 1989). Both the extracellular and membrane-bound forms of the β -lactamase of *S. griseus* NRRL B-2682 and the membrane-bound enzyme of the *spo-1* mutant were susceptible to inhibition by clavulanic acid and sulbactam, while cloxacillin had a weaker effect (data not

Fig. 4. Comparison of β -lactamase activity present in different fractions of *S. griseus* NRRL B-2682 grown at 27°C in DM liquid medium supplemented with 0.5% CA. Enzyme activities were determined at 36 h after inoculation. Total β -lactamase activity ($\square$), membrane-bound activity ($\square$) and extracellular β -lactamase activity ($\square$) of *S. griseus* in control cultures (A), in cultures supplemented with $\text{Ac}_2\text{-L-Lys-D-Ala-D-Ala}$ (B), with purified autolysin-digested cell wall fragments (C) and with penicillin-G (D). Values represent means of three independent measurements.

shown). These results show good correlation with our substrate specificity studies since it is known that clavulanic acid and sulbactam are irreversible suicidal- β -lactamase inhibitors and penicillinases have stronger affinity for clavulanic acid than cephalosporinases, whereas cloxacillin has been recognized to be a good inhibitor for cephalosporinases (Bush, 1989). Since all of the investigated characteristics of the differently localized β -lactamases were the same, we concluded that these are differently processed forms of the same protein.

Effect of globomycin on β -lactamase production of *S. griseus* wild-type and *spo-1* mutant

To verify the type of membrane anchorage of the β -lactamase, the effect of globomycin on β -lactamase secretion was examined in the wild-type strain and in the *spo-1* mutant. Globomycin ($50 \mu\text{g ml}^{-1}$) added to the DM liquid medium at 5 h after inoculation did not inhibit the growth of these strains. However, in both strains it enhanced the release of the soluble form of the enzyme compared to that of the non-treated control cultures while simultaneously reducing the amount of the membrane-bound form. The total amount of enzyme activity was not effected by globomycin (Fig. 3). In the mutant strain the time course of globomycin-induced β -lactamase secretion was very similar to that of the wild-type (Fig. 3b). The increase in β -lactamase secretion after the addition of globomycin suggests that the membrane-bound form of the enzyme possesses an N-terminal Cys-glyceride-thioether modification in *S. griseus* NRRL B-2682 (Nagarajan, 1993).

Fig. 5. Effect of penicillin-G on growth and differentiation of *S. griseus* NRRL B-2682. Cells were grown at 27 °C on DM-agar containing 0.5% CA. In the gradient plate method the lower DM-agar layer was supplemented with 100 µg penicillin-G ml⁻¹. The effect of penicillin-G was analysed at 48 h after inoculation. The white area in the centre of the plate indicates aerial mycelium and spore production.

Effect of purified autolysin-digested cell wall fragments, Ac₂-L-Lys-D-Ala-D-Ala and penicillin-G on β -lactamase production in *S. griseus* NRRL B-2682

It has been reported that β -lactamase induction is regulated by the intracellular level of mucopeptides in *E. coli* (Jacobs *et al.*, 1994, 1997). To determine the possible

involvement of peptidoglycan precursors in β -lactamase production or secretion in *S. griseus* NRRL B-2682, we examined the effect of purified autolysin-digested cell wall fragments and the cell wall analogue tripeptide Ac₂-L-Lys-D-Ala-D-Ala on β -lactamase production. In parallel experiments we also tested the effect of penicillin-G at subinhibitory concentration on β -lactamase production and secretion, since β -lactams interact with bacterial penicillin-binding proteins and thus may increase the release of murein-derived fragments. These agents were added to 5-h-old *S. griseus* NRRL B-2682 cells grown in DM liquid medium supplemented with 0.5% CA to inhibit sporulation and suppress extracellular β -lactamase production. β -Lactamase activity was measured in the culture supernatant and crude extracts of the cells, and total enzyme activity was also determined. As shown in Fig. 4, addition of purified digested cell wall fragments at 0.2 mg ml⁻¹, Ac₂-L-Lys-D-Ala-D-Ala at 0.5 mM and penicillin-G at 35 nM increased the level of secretion of β -lactamase into the extracellular fluid compared to the control cultures. At the same time these compounds reduced the amount of the membrane-bound form of the enzyme. The total β -lactamase activity remained the same in all cases.

To ensure that the increase in the amount of extracellular β -lactamase was not due to cell lysis, we also measured GS activity, which is known to be a strictly intracellular enzyme in *S. griseus* NRRL B-2682 (Penyige *et al.*, 1994), in the extracellular fluid. Our results showed that the presence of extracellular β -lactamase activity was not due to cell lysis, since GS was not detectable in the extracellular space; it was only detectable in crude cellular extracts.

Fig. 6. Agar diffusion test showing the age-dependent effect of penicillin-G on the differentiation of *S. griseus* NRRL B-2682. Cells were grown at 27 °C on DM-agar containing 0.5% CA. The control plate is shown on the left, penicillin-G-supplemented samples on the right. Paper discs containing 27 (1) and 34 nmol (2, 3) penicillin-G were placed on the agar surface 5 (1, 2) and 24 h (3) after inoculation. The photograph was taken 48 h after inoculation. The white area around the discs shows aerial mycelium and spore production.

Fig. 7. Phase-contrast micrographs of submerged cultures showing the effect of peptidoglycan fragments, analogues and penicillin-G on the differentiation of *S. griseus* NRRL B-2682 cells grown at 27 °C in DM medium supplemented with 0.5% CA. (a) Control culture in which the cells were impaired in sporulation and altered cell morphology in the presence of penicillin-G (33 nmol ml⁻¹) added to 5-h-old cells (b) and purified cell wall fragments (0.1 mg ml⁻¹) added to 5-h-old cells (c). The photographs were taken at 60 (a, b) and 72 h (c) after inoculation. Bars, 25 μ m.

Effect of penicillin-G, purified autolysin-digested cell wall fragments, Ac₂-L-Lys-D-Ala-D-Ala and adjuvant peptide (N-AcMur-L-Ala-D-isoglutamine) on the growth and differentiation of *S. griseus*

While studying the effect of penicillin-G on β -lactamase production of CA-suppressed *S. griseus* cultures, to our surprise we found that penicillin-G in concentrations

lower than its MIC (90 μ g ml⁻¹) affected the differentiation of the strain. To visualize this effect a simple gradient plate technique was used (Eisenstadt *et al.*, 1994). A slanted layer of DM-agar containing 0.5% CA supplemented with 100 μ g penicillin-G ml⁻¹ was covered with DM medium containing 0.5% CA without the antibiotic. The effect of the resulting antibiotic gradient on the morphological differentiation of the strain is shown in Fig. 5. Penicillin-G at high concentration strongly inhibited growth and spore germination (right), while at a subinhibitory concentration it restored aerial mycelium and spore production (in a narrow intensely white band, centre) and had no effect at low concentration (left) in CA-suppressed cultures.

It has been suggested that *S. griseus* becomes committed to sporulation at 16–18 h after inoculation in DM medium (Ensign, 1988). The effect of penicillin-G as a function of culture age was also examined using an agar diffusion test. Paper discs containing 27 or 34 nmol penicillin-G were placed on the DM-agar plate containing 0.5% CA at 5 and 24 h after inoculation. The morphological changes (sporulation) caused by penicillin-G were only seen when the antibiotic was applied to the culture at 5 h after inoculation; penicillin-G added to 24-h-old cells was ineffective (Fig. 6). Sporulation was confirmed by phase-contrast microscopy. The same result was obtained with ampicillin and cephaloridine (data not shown). Since *S. griseus* is able to sporulate in liquid cultures we tested the effect of penicillin-G in submerged cultures using DM medium containing 0.5% CA. At a subinhibitory concentration (33 nmol) penicillin-G counteracted the inhibitory effect of CA on the normal differentiation and restored spore formation (Fig. 7b). However, sporulation was delayed by 24 h compared to the normal life cycle. The same effect was observed after addition of 0.1 mg digested cell wall fragments ml⁻¹ (Fig. 7c) to CA-suppressed *S. griseus* cultures. In this experiment sporulation was delayed by 12 h only. Phase-contrast microscopy was also used to confirm spore formation in these cases (Fig. 7). Addition of 0.2 μ mol Ac₂-L-Lys-D-Ala-D-Ala, 0.25 μ mol adjuvant peptide or digested cell wall fragments to cells grown on DM-agar under sporulation-repressed conditions also resulted in aerial mycelium and mature spore production when these agents were added to 5-h-old cells (data not shown). In identical control experiments we have used amino acids and peptides that are not components of peptidoglycan and in those cases no effect on the differentiation process of CA-suppressed *S. griseus* cells was observed (data not shown).

DISCUSSION

In studying β -lactamase production in *S. griseus* NRRL B-2682 we have found that the wild-type strain produced a membrane-bound and an extracellular form of the same β -lactamase, while its non-sporulating *spo-1* mutant produced only the membrane-bound enzyme. Examination of the time course of β -lactamase production in cultures of the wild-type and the *spo-1* mutant showed that β -lactamase activity was first

detectable in the membrane of 5-h-old cells, just after the appearance of the germ tubes. Extracellular enzyme activity was first detectable in the late exponential phase of growth of the wild-type strain. It was low at first [$0.0006 \text{ units (mg protein)}^{-1}$], then increased about eightfold during the late exponential and early stationary phases. By contrast, the *spo-1* strain did not produce any extracellular β -lactamase and only membrane-bound activity was detectable during the whole life cycle. Addition of the autoregulator AF at a concentration of $80 \mu\text{g ml}^{-1}$ to 5-h-old *spo-1* mutant cells grown in DM liquid medium restored sporulation and resulted in the secretion of the β -lactamase into the extracellular fluid. These results suggest that the secretion-of- β -lactamase into the extracellular space is tightly coupled to the differentiation process. This observation was further supported by the fact that in all cases when cells of the wild-type strain were impaired in their ability to develop mature spores (by the addition of 0.5% CA or $15 \mu\text{M}$ *m*-APBA to submerged cultures), the secretion of β -lactamase into the medium was also inhibited.

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*.

In our experiments, globomycin, which specifically inhibits the bacterial signal peptidase II responsible for the processing of the membrane-bound lipoproteins (Hayashi & Wu, 1990), prevented the accumulation of the membrane-bound β -lactamase enzyme both in the wild-type and mutant strain. A possible explanation of this phenomenon is that in the absence of processing of the N-terminal signal peptide, due to the inhibitory effect of globomycin, the N-terminal Cys is not generated. Since this residue is the site for subsequent lipid modifications, in the absence of the available acceptor this lipid anchor is missing and as a result the unmodified β -lactamase is released into the extracellular 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 post-translationally modified membrane-bound form which is later released to the extracellular space.

In *E. coli* it was found that recycling of peptidoglycan fragments generated by cell wall turnover regulated β -lactamase gene expression (Jacobs *et al.*, 1994, 1997). In studying a possible connection between cell wall biosynthesis and β -lactamase production we have analysed the effect of purified autolysin-digested cell wall fragments, the cell wall analogue $\text{Ac}_2\text{-L-Lys-D-Ala-D-Ala}$ and penicillin-G on β -lactamase production in *S. griseus*. Our results indicated that in wild-type cultures the secretion of β -lactamase into the extracellular space was enhanced by addition of these agents to cells grown under sporulation-repressed conditions. 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 increased levels

of cell wall fragments/precursors are important factors which can affect the release of the β -lactamase from the cellular membrane.

While studying the effect of cell wall fragments, precursors and β -lactam antibiotics on β -lactamase secretion we noticed that these agents produced significant morphological changes in sporulation-repressed cultures of wild-type *S. griseus*. They overcame the inhibitory effect of CA and restored the ability of these cells to produce spores on solid medium and in liquid cultures. It is possible that increased amounts of cell wall precursors present both in the extra- and intracellular spaces influence the differentiation process of *S. griseus*, possibly by serving as a signal for initiation of the sporulation process. The restoration of the normal differentiation process was invariably coupled to the secretion of the extracellular β -lactamase enzyme.

We propose that in *S. griseus* there might be an indirect link between β -lactamase secretion and the turnover of cell wall fragments. Cell wall metabolism may provide feed-back signals to regulate differentiation and β -lactamase secretion could be a consequence of the normal sporulation process in this bacterium.

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