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Demonstration of the Presence of an Inducible β-Lactamase in *Azospirillum lipoferum*

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Abstract. *Azospirillum lipoferum* is a soil microorganism that has been shown to be resistant to penicillins. It has been suggested that resistance is due to the presence of a β-lactamase activity, but no conclusive evidence has been reported. The incubation of benzylpenicillin or nitrocephin with either whole *Azospirillum* cells or cell-free extracts was accompanied by hydrolysis of the β-lactam ring of the antibiotics. Such hydrolytic activity exhibited Michaelis and Menten-like kinetics. The enzyme was produced at a low, basal level that was increased approximately 50 times by the addition of benzylpenicillin, an increase that was completely blocked by chloramphenicol or rifampicin.

Azospirillum is a widely distributed soil bacterium living in close proximity to plant roots, where it is assumed to play an important role by virtue of its capacity to fix nitrogen and to produce plant growth substances.

So far, three species of *Azospirillum* have been recognized: *A. brasilense*, *A. lipoferum*, and *A. amazonense* [8].

Several wild-type strains of *A. brasilense* and *A. lipoferum* isolated from the roots of grasses were found to be resistant to penicillins. One of the most important mechanisms of penicillin resistance of microorganisms is the presence of β-lactamases (for a recent review, see [2]). However, the presence of these widely distributed enzymes has not yet been demonstrated in the genus *Azospirillum*. Franche and Elmerich [3], using an iodometric assay, suggested that penicillin resistance in *Azospirillum* was due to β-lactamase activity. In spite of the fact that such assay is widely used for detecting β-lactamases, a clear-cut demonstration of the presence of a β-lactamase would require the identification of the reaction products and the isolation, purification, and characterization of the enzyme.

As a first step in this direction, we report herein conclusive evidence of the presence of an inducible β-lactamase in *Azospirillum lipoferum* RG20.

Materials and Methods

Bacterial strains and media. *Azospirillum brasilense* SP7 (ATCC 29145) and *A. lipoferum* RG20 (ATCC 29708) were obtained from Johanna Döbereiner (Empresa Brasileira de Pesquisa Agropecuaria, Rio de Janeiro, Brazil); LB medium contained 10 g tryptone, 5 g yeast extract, and 5 g sodium chloride per liter.

Detection of β-lactamase activity in plates. The capacity of the microorganisms to produce β-lactamase was tested in plates by means of (i) an iodometric assay carried out in Lab Lenco Agar OXOID with 2% soluble starch, as described [1], and (ii) an acidimetric assay using Andrade's indicator, essentially as described [4]. In both cases the assay was performed in Petri dishes at 30°C for 48 h.

Cell-free extracts. Cells were harvested by centrifugation, washed in 0.1 *M* sodium phosphate, pH 7.0, resuspended in the same buffer at a concentration of $5 \cdot 10^{10}$ cells/ml, and sonicated in an MSE ultrasonic disintegrator MK2 (150 W) for 1 min at 4°C. The sonicated cell suspension was centrifuged at 12,000 *g* for 10 min to remove cell debris, and then at 103,000 *g* for 45 min to remove small membrane fragments.

Enzyme assay. β-lactamase was assayed spectrophotometrically in a Varian Cary-210, with nitrocephin 0.1 m*M* [7] as substrate in 0.1 *M* sodium phosphate buffer, pH 7.0, at 30°C. The enzymatic reaction was followed at 482 nm ($\Delta\varepsilon_{482} = 15.9\ mM^{-1} \cdot cm^{-1}$). One unit of activity is the amount of enzyme required to convert 1 μmol of nitrocephin/min at 30°C and pH 7.0.

General. Protein was determined by Lowry's method [5]. Concentration of cells was determined by measuring the absorbance

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of a cell suspension at 540 nm (A_{540} = 2 for 10^9 cells/ml). At least duplicates were carried out for the assays, and the values reported correspond to the mean of determinations that differed less than 10%.

Materials. Nitrocefin was a generous gift from Dr. C.H. O'Callaghan (Glaxo Pharmaceuticals Ltd., Greenford, Middlesex, UK).

Results and Discussion

β-lactamase production by *Azospirillum lipoferum* RG20 and *A. brasilense* SP7 was tested in plates. Both species yielded positive results, but *A. lipoferum* was shown to be a stronger producer of the enzyme. This result was in agreement with the minimal inhibitory concentration values determined with ampicillin for *A. lipoferum* (>2 mg/ml) and for *A. brasilense* (0.1 mg/ml); this suggests that resistance was closely related to β-lactamase production [3]. All the experiments reported below were carried out with *A. lipoferum*.

No β-lactamase activity could be detected in supernatants obtained by centrifugation from liquid cultures of *Azospirillum*; this indicates that the enzyme was either membrane bound or restricted to the periplasmic space, behaving in this respect similarly to other Gram-negative bacteria as *Escherichia coli* [6].

Incubation of either benzylpenicillin or nitrocephin with washed cells resulted in a change of absorption spectra of the antibiotic (Fig. 1), similar to that observed when they are incubated with known β-lactamases [7, 10]. These findings confirmed the presence of a β-lactamase activity in *A. lipoferum* cells.

When cell-free extracts were prepared as indicated in *Materials and Methods*, 100% of the activity was recovered in the supernatant of 103,000 *g*; this indicates that the β-lactamase was not tightly associated with any membrane fraction.

Preliminary kinetic studies carried out with a preparation partially purified by ammonium sulfate precipitation (50%–85% satn.) yielded linear Lineweaver-Burk plots with K_{ms} equal to 150 and 25 μ*M* for benzylpenicillin and nitrocephin, respectively. All the studies reported above have been carried out with *A. lipoferum* cells growing in a medium devoid of antibiotic. When benzylpenicillin was added to cells growing exponentially, a sharp increase in β-lactamase activity was observed after a short induction time (Fig. 2). This increase was completely blocked by inhibitors of RNA and protein synthesis,

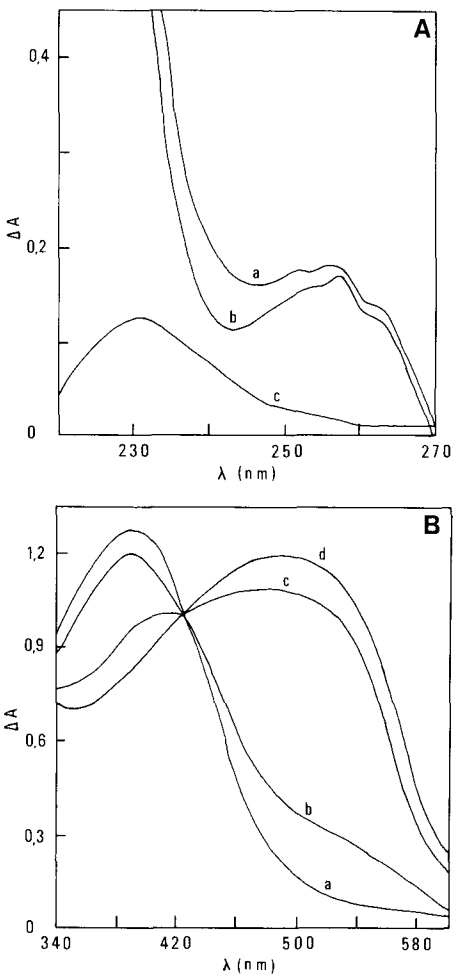

Fig. 1. Spectroscopic characterization of the products of the reaction catalyzed on β-lactam antibiotics by whole *Azospirillum lipoferum* RG20 cells. *A*: $5 \cdot 10^{11}$ cells were incubated at 30°C for 60 min in 3 ml of 0.1 *M* sodium phosphate, pH 7.0. After centrifugation, benzylpenicillin (333 μ*M*, final concentration) was added to the supernatant and the spectrum (a) was recorded in a Varian Cary 210. Spectrum (b) was recorded similarly, except that benzylpenicillin was present during the 60-min incubation. Spectrum (c) corresponds to the difference (a) − (b). *B*: 0.1 m*M* nitrocephin was incubated in sodium phosphate buffer with $5 \cdot 10^8$ cells (final volume, 3 ml), and spectra were recorded at different time intervals after the addition of cells: (a) 0, (b) 10, (c) 30, and (d) 60 min.

such as rifampicin and chloramphenicol (data not shown). This result strongly suggested that the biosynthesis of β-lactamase from *A. lipoferum* RG20 is induced by the presence of the antibiotic. Whether benzylpenicillin derepresses the biosynthesis of a single β-lactamase or induces the biosynthesis of a second and different enzyme remains to be established.

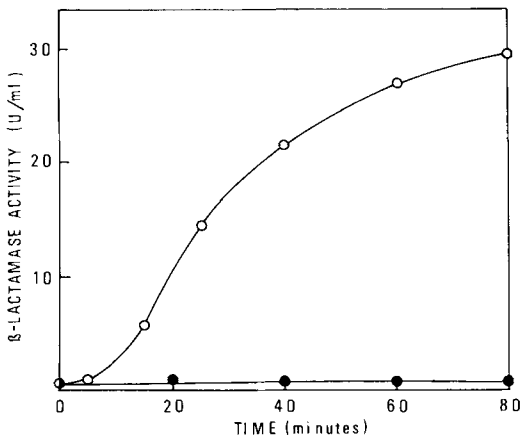

Fig. 2. Induction by benzylpenicillin of β-lactamase from *A. lipoferum* RG20. 25 μ*M* benzylpenicillin was added to half of a liquid culture in its exponential growth phase (open circles). The other half was kept as control (closed circles). At different times, aliquots were withdrawn, and protein synthesis was stopped by adding 0.1 mg/ml chloramphenicol. Cell-free extracts were prepared as indicated under *Materials and Methods* except that the centrifugation at 103,000 *g* was omitted. β-lactamase activity was determined in the supernatants as indicated in *Materials and Methods*. All the supernatants contained essentially similar protein concentration.

In summary, the production of a β-lactamase by *A. lipoferum* RG20 has been demonstrated. The enzyme, whose level depends on the presence of benzylpenicillin, is probably located at the periplasmic space and is a good candidate to explain the resistance of *A. lipoferum* to β-lactam antibiotics. For a study of the regulation and to confirm the biological role of β-lactamase in *Azospirillum*, it will be necessary to isolate mutants lacking the enzyme. Recent cloning of a gene encoding a β-lactamase from *Azospirillum* should facilitate this objective [9].

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