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Contribution of β-lactamase production to the resistance of mycobacteria to β-lactam antibiotics

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Abstract ***Mycobacterium fallax*** **(*M. fallax*) is naturally sensitive to many β-lactam antibiotics (MIC < 2 μg/ml) and devoid of β-lactamase activity. In this paper, we show that the production of the β-lactamase of *Mycobacterium fortuitum* by *M. fallax* significantly increased the MIC values for good substrates of the enzyme, whereas the potency of poor substrates or transient inactivators was not modified. The rates of diffusion of β-lactams through the mycolic acid layer were low, but for all studied compounds the half-equilibration times were such that they would only marginally affect the MIC values in the absence of β-lactamase production. These results emphasize the importance of enzymatic degradation as a major factor in the resistance of mycobacteria to penicillins.**

Key words: *Mycobacterium fallax*; Mycolic acid; β-Lactamase; Diffusion; Minimum inhibitory concentration

1. Introduction

Multiple resistance to antimicrobial agents is widely responsible for the increasing incidence of mycobacterial infections [1]. Resistance to β-lactam compounds which specifically inactivate the DD-peptidases involved in the biosynthesis of peptidoglycan [2,3] is of particular interest. In mycobacteria such a resistance is generally attributed to the permeability barrier supplied by mycolic acids which form a highly hydrophobic outer membrane-like structure. Recently, it has also been demonstrated that the cell wall of mycobacteria contains porin-like proteins which facilitate the diffusion of hydrophilic molecules [4]. Moreover, the presence of β-lactamase activity has been reported in many pathogenic mycobacteria [5] and even in the presence of β-lactamase inhibitors such as clavulanic acid, β-lactam antibiotics do not efficiently kill mycobacteria in vivo or in vitro, an observation for which three explanations are usually proposed: (i) mycobacterial β-lactamases may be insensitive to the inhibitors; (ii) the DD-peptidases may have a low affinity for β-lactam antibiotics, a phenomenon similar to that described in methicillin-resistant *Staphylococcus* and *Streptococcus* species [6]; (iii) the mycolic acid layer surrounding the peptidoglycan may act as a permeability barrier and protect the DD-peptidases [7].

The non-pathogenic *M. fallax*, isolated from the river Seine [8], is the only mycobacterial species described so far as very sensitive to ampicillin (MIC 0.1 μg/ml). Based on the 16S RNA sequence, *M. fallax* is phylogenetically close to *M. chitae*, *M. chelonae* and *M. fortuitum*. This nonchromogenic species grows rapidly at 30°C but slowly at 37°C. Interestingly, *M. fallax* is resistant to most conventional antituberculosis drugs, including isoniazid (> 1 μg/ml), *para*-aminosalicylic acid (PAS) (> 10 μg/ml), streptomycin (> 10 μg/ml) and rifampicin (> 20 μg/ml) [9].

In this report, we show that a low permeability coefficient is not sufficient to induce resistance but that the production of β-lactamase can be a determining factor in this phenomenon.

2. Materials and methods

2.1. Bacterial strains, plasmid and culture conditions

M. fallax is naturally sensitive to β-lactam antibiotics and devoid of β-lactamase activity. Using the procedure described for *M. smegmatis* mc²155 [10] this strain was transformed by electroporation with the *Escherichia coli*/*Mycobacterium* pIPJ42* shuttle plasmid carrying a pblaF*-blaF fusion [11] which allows the overproduction of the *M. fortuitum* β-lactamase, yielding the *M. fallax* pIPJ42* strain.

M. fallax was grown at 30°C on Müller-Hinton agar added with 0.2% glycerol. For liquid cultures, Müller-Hinton broth was supplemented with 0.1% Tween 80 and 0.2% glycerol. Kanamycin (20 μg/ml) or ampicillin (50 μg/ml) were added in cultures of the transformed strain.

2.2. Antibiotics

Kanamycin sulfate and imipenem were gifts from Merck Sharp and Dohme Laboratories (Rahway, NJ, USA). Other compounds were obtained as before [12].

2.3. Kinetics of extracellular and cell-bound β-lactamase production

Precultures (100 ml) of the two *M. fallax* strains were grown for 72 h at 30°C under orbital agitation. 1 ml of the preculture was added to 300 ml of fresh medium and the culture continued under the same conditions. Aliquots were withdrawn at 6 h intervals.

The progress of the culture was monitored by determining the dry weight and the A_{600} values. The β-lactamase production was measured as follows: 5 ml aliquots were centrifuged at 13 000 × *g* for 5 min. The supernatant was kept on ice and the pellet resuspended in 300 μl of 60 mM sodium phosphate buffer pH 7.3, sonicated (three cycles of 30 s) and centrifuged at 13 000 × *g* for 20 min in order to separate the membrane fraction from the soluble material containing the periplasmic and cytoplasmic compartments. The β-lactamase activity was assayed by measuring the initial rate of hydrolysis of a 100 μM solution (500 μl) of nitrocefin in 60 mM phosphate buffer pH 7.3.

The presence of the β-lactamase in the different cellular compartments was detected with the help of rabbit antibodies raised against

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Abbreviations: MIC, minimum inhibitory concentration; PBP, penicillin binding protein

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the *M. fortuitum* β-lactamase. 10 μg of protein of the different fractions was loaded on 12% SDS-PAGE gels (10×8×0.75 cm, BioRad). The proteins were transferred onto Immobilon-P membranes and the enzyme detected by Western blotting, followed by 5-bromo 4-chloro 3-indolyl phosphate revelation according to the Bio-Rad protocol.

2.4. Properties of the β-lactamase

The steady-state kinetic parameters were determined with enzyme produced by *M. smegmatis* after transformation with pIPJ42* and purification to homogeneity as described before [12].

The measurements were performed at 30°C in 50 mM sodium phosphate (pH 7.4) containing 20 μg/ml of bovine serum albumin and 0.2 M KCl. Values represent averages of five different experiments performed at various concentrations. The data were collected with a Uvikon 80 spectrophotometer linked to a Copam microcomputer via a RS232C interface and analyzed as before. With nitrocefin the k_{cat} and K_m values were obtained by complete time-course analysis. The other compounds were used as inhibitors and the K_m derived as K_i values. Subsequently, k_{cat} was estimated by determining the initial rate at substrate saturation. With oxacillin and cefoxitin, pseudo-first order rate constants for accumulation of acylenzyme were determined by the reporter substrate method.

On the basis of the three-step model

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_2} ES^* \xrightarrow{k_3} E + P$$

where ES* is the acylenzyme and $K' = (k_{-1} + k_2)/(k_{+1})$, the values of k_2/K' ($= k_{cat}/K_m$) and k_3 were determined as described by Galleni et al. [14]. That k_3 was equal to k_{cat} was shown again by monitoring the hydrolysis of a 1 mM solution of the compounds [13].

2.5. Determination of the MICs

When liquid cultures reached an A_{600} value of 0.6, an aliquot was diluted 10^5-fold in phosphate buffer and 100 μl streaked on agar plates containing increasing concentrations of β-lactam antibiotics or kanamycin. The plates were incubated at 30°C and the results recorded after 5 days.

2.6. Assay of M. fallax cell wall permeability towards β-lactam antibiotics

Cell wall permeability was determined by comparing the rates of hydrolysis of the β-lactam compound by intact and sonicated cells [15]. The cells from a 350 ml culture were centrifuged, washed once in 60 mM phosphate buffer, pH 7.3, supplemented with 0.2 M NaCl and 1 mM phenylmethylsulfonyl fluoride (PMSF) and resuspended in 35 ml of the same buffer. The dry weight of an aliquot was also measured. The cell suspension (100 μl) was added to 10 ml of antibiotic solution at different concentrations prepared in the same buffer. The other experimental conditions are described in Table 1. At 15 min intervals, aliquots (1 ml) were filtered through 0.22 μm filters. The concentration of intact antibiotic was determined by UV spectroscopy at a wavelength specific to the drug (penicillins: 230 nm; nitrocefin: 482 nm; other cephalosporins: 260 nm) [12]. Similarly, the rate of hydrolysis was measured after sonication (3×1 min at 0°C) of the cell suspension. This resulted in a disruption of 95% of the cells as verified by microscopy.

The permeability coefficient was calculated by the method of Zimmermann and Rosselet [15] adapted for mycobacteria by Jarlier and Nikaido [16]. The surface/weight ratio of *M. fallax* (132 cm^2/mg dry weight) was assumed to be similar to that of *Salmonella typhimurium*, an organism of similar size and shape.

3. Results

3.1. β-Lactamase production by M. fallax pIPJ42* and kinetic parameters

Competent cells of *M. fallax* were prepared from an exponentially growing culture. The transformation yield with pIPJ42* was very low (5–15 transformants per μg of DNA) and attempts to create an hypercompetent mutant of *M. fallax* remained unsuccessful. The plasmid content of the transformants was verified by electroduction in *E. coli* [17]. The production of the β-lactamase by the transformants was constitutive. Most of the β-lactamase remained cell-bound during the logarithmic growth phase (Fig. 1). Less than 20% of the enzyme was released in the medium. These results were confirmed by a Western blot (Fig. 2). A large proportion of the β-lactamase was found to be cell-bound, in good agreement with the observation reported by Wagner et al. [18].

The catalytic properties of the purified enzyme were very similar to those of the original enzyme produced by *M. fortuitum* itself (Table 1). Ceftazidime was not recognized by the enzyme. Benzylpenicillin, ampicillin, cefuroxime, cephaloridine, carbenicillin and cefotaxime were rather good substrates while cefoxitin, imipenem and oxacillin acted as poor transient inactivators due to slow hydrolysis and accumulation of the acylenzyme (low k_3 values).

3.2. MICs of M. fallax with and without pIPF42*

The impact of the production of the *M. fortuitum* β-lactamase on the MIC values of *M. fallax* is reported in Table 1. The MIC values barely increased with oxacillin and remained unchanged with ceftazidime, cefoxitin and imipenem. The antibiotic potency of these poorly recognized compounds is thus directly correlated to their affinity for the essential PBPs and their relative diffusion rate through the mycolic acid layer. Ceftazidime was rather inefficient when compared to imipenem and cefoxitin.

Table 1
MIC values for *M. fallax* strains with and without β-lactamase and kinetic parameters for the interaction between the *M. fortuitum* β-lactamase produced by *M. fallax* and β-lactam antibiotics

Antibiotic	MIC (μg/ml)				
	M. fallax	*M. fallax* pIPJ42*	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
Benzylpenicillin	<2	40	50±5	30±3	1000
Ampicillin	0.125	48	200±20	60±9	300
Ceftazidime	75	75	>1000	ND	ND
Oxacillin	20	40	170	$k_3 = 0.048 \pm 0.005$	0.3
Cefuroxime	<1.3	26	96±10	6±0.6	60
Cephaloridine	<1.5	30	125±12	24±3	200
Carbenicillin	9.5	125	75±7	3±0.3	40
Cefotaxime	<1.5	30	55±5	2±0.2	40
Imipenem[a]	<2	<2	530	0.16±0.01	0.3
Nitrocefin	ND	ND	160±16	450±45	2000
Cefoxitin	<2	<2	100	$k_3 = 0.015 \pm 0.002$	0.016

[a]Value for the very similar *M. smegmatis* β-lactamase.
ND = not detectable.

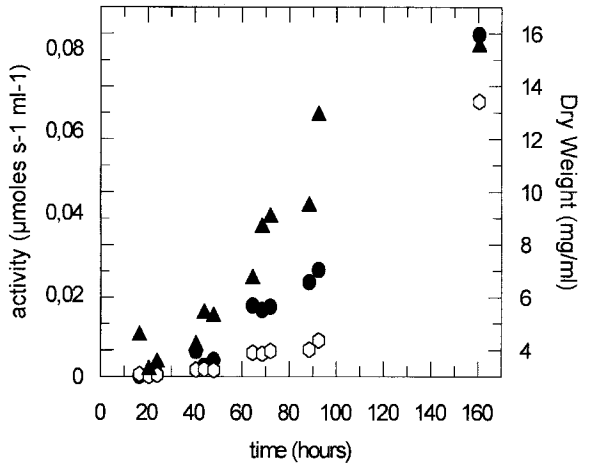

Fig. 1. Growth of *M. fallax* pIPJ42* strain (dry weight, ▲) and production of secreted, soluble (○) and cell-bound β-lactamase (●). The enzyme activity was measured as the rate of hydrolysis of nitrocefin in µmol ml^{-1} min^{-1}.

The efficiency of all the other tested compounds, which were good substrates for the enzyme, sharply decreased in the presence of β-lactamase (MIC > 20 µg/ml).

3.3. Permeability of the cell wall of M. fallax

The data were obtained with a culture of *M. fallax* pIPJ42* in the logarithmic phase, where most of the β-lactamase was cell-bound. With intact cells, less than 1% of the activity was released in the supernatant during the assay and the rates of hydrolysis of β-lactam compounds were 3–45% of those observed with the sonicated extracts. The permeability coefficients were measured at different concentrations of antibiotics. The small variation in the calculated values indicated that artefacts due to the nonspecific binding of the antibiotics to the cell surface or to cellular debris could be excluded.

The permeability coefficient P ranged from 2.3 nm/s for cefotaxime to 66 nm/s for nitrocefin indicating a relatively low permeability of *M. fallax* to all tested compounds when compared to *Pseudomonas aeruginosa* and *E. coli* [16] (Table 2).

4. Discussion

The morphology of *M. fallax* is very similar to that of *M. tuberculosis*. Its cell wall contains tuberculostearic acid and di- and tri-unsaturated α-mycolic acids [20], a pattern which had not been previously described in mycobacteria. However, there is no evidence that the *M. fallax* cell wall exhibits specific permeability properties. As shown in Table 2, the permeability coefficients were significantly larger than for *M. chelonae* [16], but quite similar to those observed for *M. tuberculosis* [19]. The permeability coefficient for nitrocefin was somewhat lower than for *E. coli* (200 nm/s) but 10-fold larger than for *P. aeruginosa* (5 nm/s) whereas that for cephaloridine was similar for both *P. aeruginosa* and *M. fallax* [20,21]. Several compounds (cefotaxime, cefuroxime, benzylpenicillin and ampicillin) exhibited low diffusion rates, at least 3 orders of magnitude lower than those observed for *E. coli*. With the cephalosporins, the permeability coefficient of *M. fallax* appeared to be grossly correlated to the hydrophobicity of the tested compound. A similar situation appeared to prevail with the two studied penicillins. However, these latter compounds exhibited lower diffusion rates than cephalosporins of similar log Pu. Although other factors might be involved, as indicated by the difference between penicillins and cephalosporins, these data suggest a simple diffusion process through the mycolic acid layer, a situation which is in contrast to that observed in enterobacteria and *Pseudomonas*, where porin-mediated diffusion consistently favors the more hydrophilic compounds. These results demonstrate that a low permeability coefficient does not by itself account for the resistance of mycobacteria. Indeed, due to the low growth rate of *M. fallax*, even those compounds which diffuse slowly exhibit a relatively short half-equilibration time (2.4 h for cefotaxime) when compared to the generation time observed here (13 h). In consequence, in the absence of β-lactamase production, a 2 µM external concentration of cefotaxime would result in a periplasmic concentration of 1.5 µM after 5 h.

Under these conditions, even with a very low acylation rate constant of 100 M^{-1} s^{-1} for an essential PBP, 80% of the target enzyme would be inactivated after an additional 2.8 h. Note that 100 M^{-1} s^{-1} is only 6–10-fold higher than the value measured for the acylation of the 'penicillin-resistant' PBP 2a of the methicillin-resistant *Staphylococcus aureus* by benzylpenicillin. Even with a lower generation time of 3–5 h, the ratio of external to periplasmic concentration of β-lactam would not exceed a factor of 3–4 during exponential growth. In consequence, it is not surprising that the MIC values for *M. fallax* with many of the β-lactams studied here were below 2 µg/ml (about 5 µM) and these MIC values probably reflect the sensitivity of the most sensitive essential PBPs. In this respect, ceftazidime, oxacillin and, to a somewhat lesser degree, carbenicillin appear to behave as poor acylating agents for these enzymes. The production of a β-lactamase by strain pIPJ42* drastically modified the situation. Indeed, if the MIC values for the poor substrates ($k_{cat}/K_m < 500$ M^{-1} s^{-1}) remained similar to those observed for the strain without β-

Table 2
M. fallax cell wall permeability to β-lactam antibiotics

Antibiotic	Concentration (µM)	Dry weight (mg/100 µl)	Permeability coefficient (nm/s)			Net charge at neutral pH	Hydrophobicity (log Pu) [25]
			M. chelonae [19]	*M. tuberculosis* [19]	*M. fallax*[a]		
Cephaloridine	41–82	5.2	1.0	9.4	27.3	Zwitterion	2.04
Cefotaxime	46–76	5.2			2.3	–	−1.05
Cefuroxime	62–74	5.2			6.4	–	−0.16
Nitrocefin	155–164	5.2	0.79		66	–	2.1
Penicillin G	470–1180	4.05		4.9	6	–	1.7
Ampicillin	312–650	4.05		3.1	3.5	–	0.96

[a]S.D. values are ± 10%.

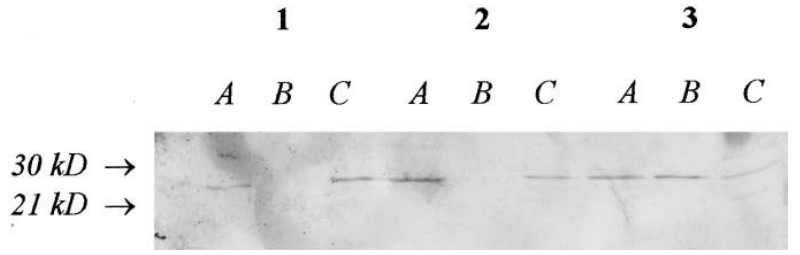

Fig. 2. Distribution of the β-lactamase in the different cell compartments in function of the cell growth. 1, 2 and 3 represent aliquots withdrawn after 65, 90 and 160 h respectively. A, B and C represent the culture medium, the 'periplasm'/cytoplasm and the cell wall/membrane fractions respectively. The protein was visualized with the help of antibodies raised against the *M. fortuitum* β-lactamase.

lactamase, those for the other antibiotics were very sharply increased. The *M. fortuitum* β-lactamase exhibits a rather broad spectrum of activity, including cefuroxime and cefotaxime among its relatively good substrates. Accordingly, the MIC values for compounds with k_{cat}/K_m values above 1000 M^{-1} s^{-1} increased at least 20-fold. During exponential growth, the enzyme was mainly cell-bound, and found in the membrane and periplasmic fractions. During the stationary phase, more enzyme was secreted into the medium and the situation thus appears to be intermediate between those observed with Gram-negative (periplasmic β-lactamase) and Gram-positive (membrane-bound and extracellular enzymes) bacteria.

In conclusion, it does not seem that the diffusion barrier due to the mycolic acid layer can, per se, greatly affect the sensitivity of *M. fallax* to β-lactam antibiotics. However, in combination with the production of a β-lactamase, a slow diffusion rate can prevent the lethal action of these compounds and the presence of a broad spectrum enzyme becomes the major cause of resistance of the strain. In consequence, the exact contribution of the β-lactamase to the resistance of pathogenic mycobacterial species might be more important than previously expected and needs to be carefully re-evaluated. For example, allelic exchange may be used to knock out the gene coding for β-lactamase as described for the urease locus in *M. bovis* BCG [26]. Although the mycobacterial β-lactamases studied so far are class A enzymes [12,22], they are relatively insensitive to clavulanic acid, and this probably accounts for the inefficiency of this inhibitor which has been quite useful when administered in combination with amoxycillin or ampicillin in the case of TEM or SHV β-lactamase producing strains.

The interaction between β-lactams and the PBPs of *M. fallax* is currently under investigation. This should allow the building of a mathematical model to explain the resistance of mycobacteria to these antibiotics, an approach which has been successfully utilized in the study of β-lactamase producing Gram-negative strains [23,24].

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