The Target of Penicillin

The Murein Sacculus of Bacterial Cell Walls Architecture and Growth

Proceedings
International FEMS Symposium
Berlin (West), Germany, March 13–18, 1983

Editors
R. Hakenbeck · J.-V. Höltje · H. Labischinski

Walter de Gruyter · Berlin · New York 1983

FUNCTION OF PENICILLIN-BINDING PROTEIN 3 IN *STREPTOCOCCUS FAECIUM*

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Introduction

The membranes of *Streptococcus faecium* (*S. faecalis* ATCC 9790) contain a maximum of eight penicillin-binding proteins (PBPs) (1). Among them, only the protein with the smallest molecular weight (43,000 Mr) has been shown to possess an enzymatic activity. It is a DD-carboxypeptidase (2). Up to now, no physiological function could be attributed to any of these membrane proteins.

The results presented in this paper tend to show that PBP3 is involved in cell septation and PBP2 and/or PBP6 are implicated in pole formation.

Results

At 37°C in a rich medium, cefotaxime, a recently introduced cephalosporin (3), has the same inhibitory potency on *S. faecium* as benzylpenicillin (MIC ≅ 4 μM) but these two β-lactams have different MBC values : 420 μM for cefotaxime and 8 μM for benzylpenicillin. Hence, under these growth conditions, *S. faecium*

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shows tolerance for cefotaxime. When the growth temperature is reduced (32°C), the MIC value of cefotaxime is increased to 1000 µM and the tolerance phenomenon disappears. Under identical conditions, benzylpenicillin shows almost unchanged MIC and MBC values (8 and 16 µM , respectively).

At 37°C in a rich medium, cefotaxime, at the MIC, inhibits completely *S. faecium* growth only 2 hrs after the beginning of the treatment. The inhibition is temporary and growth resumes. At a concentration corresponding to 20 × MIC, cefotaxime induces a slow lysis.

Morphology is deeply influenced by cefotaxime treatment. At concentrations around the MIC (5 µM) or below (0.1 to 1 µM), the normal coccal cells (Fig. 1,A) are transformed in bacilliform cells whose length increases as the duration of the treatment increases (Fig. 1,B). The diameter of the treated cells is also increased. Thin sections of such cells, examined by transmission electron microscopy, show that septation is inhibited (Fig. 2). Septa are initiated but never reach completion. In addition, constrictions similar to those seen in normal cells (Fig. 3) are not observed.

Affinity of the PBPs (of isolated membranes and intact cells) for cefotaxime has been determined by direct binding experiments using [^{14}C]cefotaxime or by competition experiments between non radioactive cefotaxime and radioactive [^{14}C]benzylpenicillin. Affinity is defined as the concentration permitting 50 % saturation. Both techniques yield essentially the same results (Table 1). Cefotaxime binds preferentially to the three highest molecular weight proteins. PBPs 2 and 3 are about 100-fold more sensitive than PBP1 and therefore must be involved in cell septation. This is confirmed by time course experiments using cefotaxime at subinhibitory concentration (1 µM). From cell samples, collected 30 and 60 min after addition of cefotaxime, membranes are isolated, labelled with saturating [^{3}H]benzylpenicillin and examined by fluorography (Fig. 4,B). Under these conditions, only PBPs 2 and 3 are saturated by

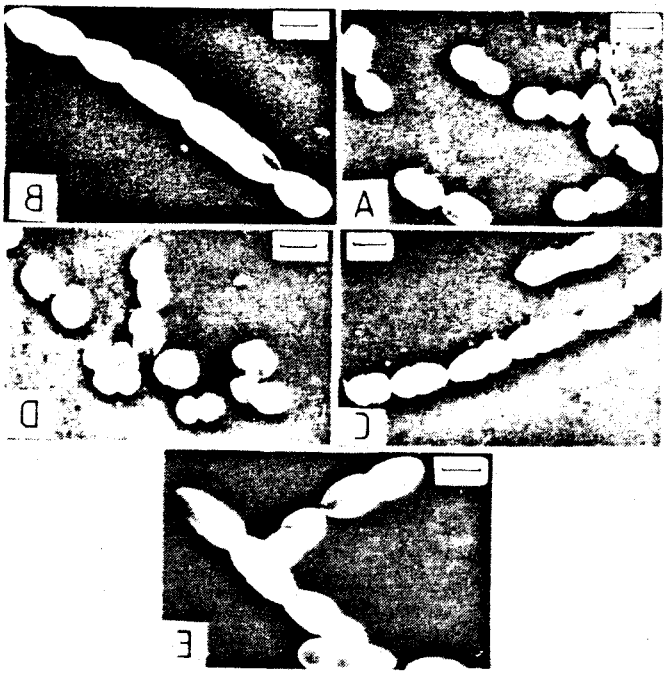

Figure 1. Scanning electron micrographs of untreated wild type cells (A); cefotaxime-treated (120 min) wild type cells (B); cefoxitin-treated (120 min) wild type cells (C); untreated $NT_1/20$ mutant cells (D); cefotaxime-treated (120 min) $NT_1/20$ mutant cells (E). The bar represents 1 µM .

Table 1. Affinity of the PBPs

PBPs	Cefotaxime	Cefoxitin	Benzylpenicillin
1	11.0	4.8	0.4
2	0.05	0.05	0.24
3	0.13	3	0.08
4	1300	215	1.2
5	—	85	> 10
4*	1300	—	1.0
6	2500	0.50	1.3

Results expressed as the concentrations (µM) required to achieve 50 % binding to the proteins. They represent the mean value of several experiments.

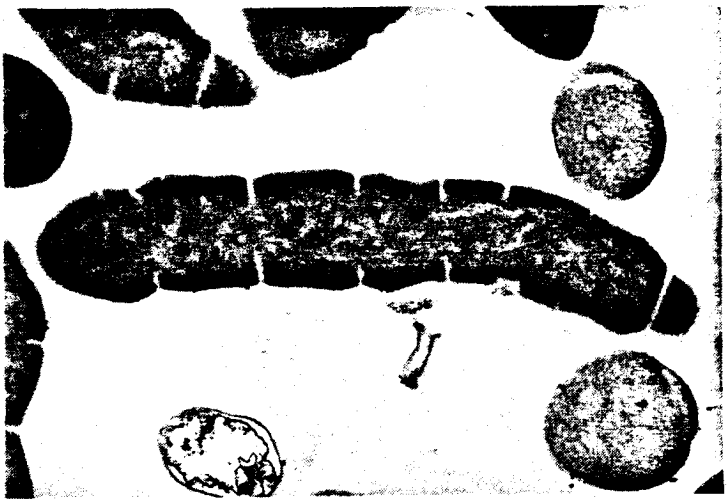

Figure 2. Thin sections of cefotaxime treated (90 min) wild type cells of *S. faecium*.
The bar represents 1 µM .

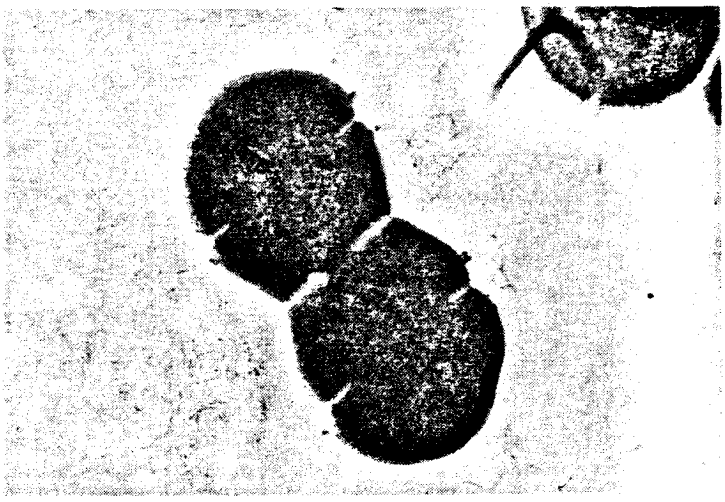

Fig. 3. Thin sections of untreated wild type cells of *S. faecium*.
The bar represents 1 µM .

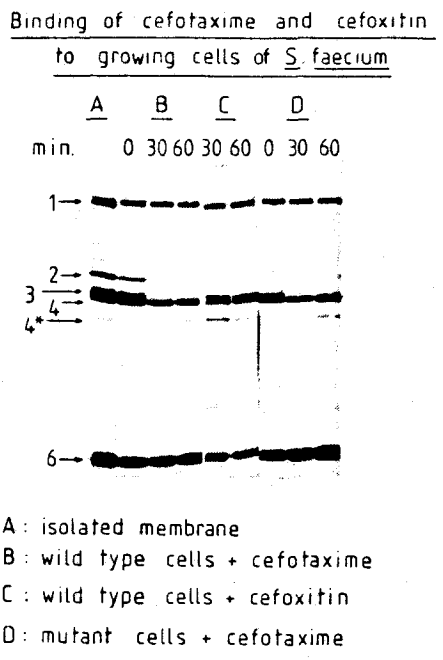

Figure 4. A : isolated membranes; B : wild type cells + cefotaxime; C : wild type cells + cefoxitin ; D : $NT_1/20$ mutant cells + cefotaxime.

cefotaxime. At this stage, no distinction can be made between the two proteins; either both or only one of them may be involved in cell division.

As shown by binding experiments, carried out on both isolated membranes (1) and intact cells (Table 1), PBP2, PBP6 and then PBPS 3 and 1 have, in the indicated order, decreasing affinities for cefoxitin. PBPs 5 and 4 are the most resistant ones. The MIC and MBC values are 50 and 500 µM , respectively (1). At the MIC, cefoxitin inhibits completely cell growth and induces a very slow lysis. Below the MIC, growth inhibition decreases gradually as the antibiotic concentration decreases. Above the MIC, cell lysis is more rapidly induced as the cefoxitin concentration increases.

The morphological abnormalities induced by cefoxitin, at a concentration (0.5 µM) below the MIC, differ from those induced by cefotaxime (1 µM). As shown by scanning and transmission electron microscopy, the cefoxitin-treated cells have an increased diameter and are slightly elongated (Fig. 1,C). But the most stricking alteration is the frequent presence of conical poles contrasting with the round poles observed in control cells (Fig. 1,A).

Analysis of membranes isolated from cells grown for 30 and 60 min in the presence of a subinhibitory concentration of cefoxitin (0.5 µM) shows that PBPs 2 and 3a are completely saturated while PBP6 is partially saturated. The other PBPs are not affected. Thus, the morphological alterations observed in cefoxitin-treated cells can be attributed only to the inhibition of the functioning of one or several of these 3 PBPs.

Elongated cells similar to those obtained with cefotaxime are never found in cultures treated with cefoxitin at concentrations sufficient to saturate PBP2. As already suggested above, PBP6 does not play any direct role in the inhibition of cell septation. The main difference between cefotaxime- and cefoxitin-treated cells is that PBP3 is saturated by cefotaxime but completely untouched by cefoxitin. Thus, septation inhibition must be due to the interaction of cefotaxime with PBP3.

A constitutive ($NT_1/20$) mutant of *S. faecium* devoid of PBP2 grows at 32°C more slowly than the wild type strain (generation times : 42 and 36 min , respectively). At 37°C, the generation time is 60 min and the mutant does not grow at all at 42°C. It may be a double mutant because revertants capable of growing at 42°C as the wild type does have been isolated. They still lack PBP2. At 32°C, the $NT_1/20$ mutant has a normal morphology but it is slightly smaller than the wild type cells (Fig. 1,D). Thus the absence of PBP2 seems not to cause any important morphological alteration. Consequently, PBP2 appears not to have any vital function in *S. faecium*.

When treated with cefotaxime (1 μM), the $NT_1/20$ mutant grows as elongated cells similar to those observed with wild type treated cells (Fig. 1,E). Analysis of the PBPs in cefotaxime-treated cultures of the $NT_1/20$ mutant shows that only PBP3 is saturated by the antibiotic and is uncapable of binding [^{3}H]benzylpenicillin (Fig. 4,D).

Discussion

PBP3 is critical for cell division probably at the level of cell septation. Its function can be related to that of PBP3 in *Escherichia coli* (4) which is a DD-transpeptidase specifically required for cell septation (5).

PBP2 has no obvious role although it may be somehow involved in cell size control. Its function could be taken over by one or several of the other PBPs (6). When the functioning of PBP2 and/or PBP6 is inhibited by a β-lactam such as cefoxitin, cells of *S. faecium* are induced to form conical poles.

Acknowledgements

This work has been supported in part by the US National Institutes of Health (grant AI 13364-05); the FRSM, Brussels (grant 3.4501-79), the Belgian Government (*Action concertée* 79/84-I1) and the FNRS, Brussels (*crédit aux chercheurs*).
The $NT_1/20$ mutant is a gift of Dr. P. Canepari from the Istituto di Microbiologia, Università di Genova, Genova, Italy.

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