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Penicillin binding protein 2x as a major contributor to intrinsic β-lactam resistance of *Streptococcus pneumoniae*

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The production and purification to protein homogeneity of a soluble form of PBP2x from a cefotaxime-resistant *Streptococcus pneumoniae* strain is reported. It was obtained by a site-directed deletion of the membrane anchor in the corresponding gene, a method similar to that successfully utilized for the production of PBP2x from a cefotaxime-sensitive wild type strain [1]. The kinetic parameters characterizing the interactions of both cefotaxime-resistant and -sensitive proteins have been determined and compared. The results are in agreement with the identification of PBP2x as the primary target for cefotaxime in the sensitive strain and as probably one of several targets in the resistant strain.

Penicillin binding protein; Penicillin; Resistance, Streptococcus

1. INTRODUCTION

The targets of β-lactam antibiotics are membrane-bound enzymes usually called PBPs (for penicillin binding proteins) involved in the biosynthesis and remodelling of the bacterial cell wall peptidoglycan. They catalyse transpeptidation reactions which result in the crosslinking of the glycan chains by short peptides. Penicillin and other β-lactams inactivate these enzymes by acylating the active site serine residue involved in the transpeptidase reaction [2,3]. PBPs sometimes also contain a second domain responsible for catalysing the elongation of the glycan chains (transglycosylation).

In both Gram-positive and Gram-negative bacteria intrinsic β-lactam resistance has been associated to the appearance of high molecular weight penicillin binding proteins (HMW PBPs) which exhibit a reduced 'affinity' for the β-lactam compounds [4].

The worldwide emergence of penicillin-resistant strains of *Streptococcus pneumoniae* is a crucial problem in human antibacterial therapy [5]. In this bacterium, alteration of target enzymes is the main mechanism responsible for the development of penicillin resistance [6–8].

S. pneumoniae contains five HMW PBPs (1a, 1b, 2x, 2a and 2b) [9] and a LMW PBP (3) [10]. PBP2x appears to be involved in the β-lactam resistance since it is the first PBP to be altered in cefotaxime-resistant laboratory mutants [11] and it is also modified in clinical isolates [12]. β-Lactam resistance in both laboratory mutants and natural populations is acquired by a stepwise alteration of the PBPs [11,13] although completely different mechanisms seem to be involved [12,14]. Reduction of β-lactam binding in four essential PBPs (1b, 2x, 2a and 2b) has been shown to occur in β-lactam resistant strains. In clinical isolates, the variations of the PBP electrophoretic patterns and the discovery of 'mosaic' PBP gene structures reveal an extensive remodelling of the penicillin binding domain of different PBPs. These modifications probably occurred by gene transfer and subsequent recombinational events.

Acquisition of β-lactam resistance in laboratory mutants involves point mutations [15] which successively appear in one or several PBPs and which each contribute to the increase of the resistance level of the strain. Several low-level cefotaxime-resistant variants have been isolated which exhibited a few amino acid substitutions in the penicillin binding domain of PBP2x [16]. For instance, the C604-PBP2x variant obtained after six selection steps displayed three amino acid substitutions in the penicillin binding domain (T 550 A, S 596 L and G 601 E). The sequences of mutational events revealed a complex process of resistance development since different PBPs are concerned and similar levels of resistance can be acquired with totally different mutation patterns.

Like other HMW PBPs, PBP2x is bound to the cytoplasmic membrane by a short N-terminal hydrophobic peptide [15]. By site-directed deletion of this membrane anchor, the protein was produced in a soluble form (called PBP2x*) and purified [1]. This derivative was highly soluble and stable, which greatly facilitated its purification and detailed study. It catalysed both the

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hydrolysis and aminolysis of different ester and thiolester substrates and the kinetic parameters characteristic of their interactions with β-lactam compounds could easily be determined [17].

In this paper, we report the production and the purification of a soluble PBP2x derivative (PBP2x*) from the cefotaxime-resistant C604 mutant and a comparison of its kinetic parameters with those of the cefotaxime-sensitive R6 strain PBP2x*. We also discuss the correlation between the observed variations and the 'minimum inhibitory concentration' values.

2. MATERIALS AND METHODS

2.1. *Plasmids*

The pCG31 plasmid encoding PBP2x* from the R6 penicillin-sensitive strain was obtained by site-directed deletion of the membrane anchor using polymerase chain reaction technology [1]. A plasmid encoding a soluble form of PBP2x from the C604 mutant was constructed by replacing the 1.8 kb *Pst*I–*Eco*RI fragment with the corresponding fragment of the C604-PBP2x gene

2.2. *Enzymes and chemicals*

The soluble PBP2x derivatives from the penicillin-susceptible (R6) and penicillin-resistant (C604) strains were produced in *E. coli* DH5α and purified according to Jamin et al. [17]. These proteins are referred to as R6-PBP2x* and C604-PBP2x* in the following text.

The β-lactamase from *Bacillus licheniformis* was produced and purified as described by Matagne et al. [18].

Benzylpenicillin was from Rhône-Poulenc (Paris, France), ampicillin and oxacillin were from Bristol Benelux (Bruxelles, Belgium), carbenicillin, methicillin, temocillin and ticarcillin were from Beecham Research Laboratories (Brentford, Middx., UK), cefotaxime was from Hoechst-Roussel (Romainville, France), cephalexin, cephaloglycin and cephaloridine were from Eli Lilly and Co. (Indianapolis, IN, USA), piperacillin was from Cyanamid and cefoxitin was from Merck Sharp & Dohme (Research Laboratory, Rahway, WJ, USA). All antibiotics were kindly given by the respective companies. Nitrocefin was purchased from Oxoid (Basingstoke, Hants., UK) The depsipeptide substrates, hippuryl-phenyllactic acid (S1e), hippuryl-thiolactic acid (S2c) and benzoyl-D-alanyl-thioglycolic acid (S2d) were prepared as previously described [19,20] The structures of these compounds can be found in the same articles.

2.3. *Determination of kinetic parameters and MICs values*

All the kinetic parameters were determined at 37°C in 10 mM sodium phosphate buffer pH 7.0 as described in Jamin et al. [17]. In brief, the hydrolysis of the substrates was directly monitored at 250 nm, the rate of acylation by β-lactams was determined by following the time-dependent fluorescence decrease reflecting the accumulation of acyl-enzyme (λ_{exc} = 290 nm, λ_{em} = 340 nm) and the rate of deacylation by recording the recovery of the enzymatic activity after elimination of the excess of inactivator by addition of the *B. licheniformis* β-lactamase

The determination of the minimum inhibitory concentration values (MICs) was as described in Laible et al. [11]. The MIC value represents the concentration of antibiotic which is necessary to prevent visible growth starting with a sample containing 1×10^5 cells ml^{-1}, thus supplying an estimate of the susceptibility of the bacteria to the antibiotic.

3. RESULTS AND DISCUSSION

Three direct isoforms of the C604-PBP2x* were separated on the Mono S column representing respectively 25, 45 and 30% of the total protein. Each fraction yielded a single band upon SDS-PAGE. The rates of acylation of the three isoforms by benzylpenicillin were not significantly different, indicating similar enzymatic properties. Such a situation had previously been found with the cefotaxime-sensitive R6-PBP2x*. In both cases, the experiments were performed with the second, most abundant isoform.

The concentration of the R6-PBP2x* was determined by active-site titration with benzylpenicillin [17]. Unfortunately, the low activity of the C604-PBP2x* did not allow the utilization of this method for the modified enzyme. In consequence, the concentration of the C604-PBP2x* in the different Mono-S-fractions was estimated by fluorography after labelling with ^{14}C-benzylpenicillin. The intensity of the bands was compared to that obtained with a known amount of the R6-PBP2x* and indicated stoechiometric binding of the label to the protein. It could thus be considered that each of the isoforms was purified to homogeneity.

Intrinsic resistance phenomena raised the intriguing questions of whether or not the mutations inducing alterations of the pencillin-binding properties of the PBPs also had consequences on the efficiency of the utilization of the physiological substrates by these enzymes and if the modified PBPs were capable of fulfilling their role in peptidoglycan synthesis. In this respect, the hydrolytic activity of the modified PBP2x* from the C604 strain was determined vs. three substrates and the following k_{cat}/K_m values were obtained: hippuryl-phenyllactic acid (S1e): $k_{cat}/K_m = 60 \pm 20\ M^{-1} \cdot s^{-1}$, hippuryl-thiolactic acid (S2c): $k_{cat}/K_m = 350 \pm 100\ M^{-1} \cdot s^{-1}$ and S2a) and benzoyl-D-alanyl-thioglycolic acid (S2d): $k_{cat}/K_m = 350 \pm 50\ M^{-1} \cdot s^{-1}$. With benzoyl-D-alanyl-thioglycolic acid (S2d), aminolysis by D-alanine was also observed as shown by the HPLC detection of benzoyl-D-Ala-D-alanine. The kinetic parameters k_{cat}/K_m for the hydrolysis of the thiolester substrates were thus decreased about ten-fold when compared to the R6-PBP2x*. However, the previous studies performed with soluble DD-peptidases indicate that point mutations do not necessarily have the same consequences on the behaviour of these esters and thiolesters and on that of the peptides which are more similar to the natural substrates [21,22]. Nevertheless, the enzymatic activity of the cefotaxime-resistant PBP was helpful for the determination of the kinetic parameters characteristic of the interaction with β-lactams.

In a previous report, it was shown that accumulation of the acyl-enzyme with PBP2x* resulted in a quenching of the intrinsic fluorescence of the protein [17]. A similar time-dependent quenching was observed with the C604-PBP2x* and thus, the k_2/K parameters could easily be determined for both enzymes and with various β-lactam compounds. Table I compares the values obtained with the R6 and C604 enzymes. The deacylation rate constants (k_3) which were measured by following the recovery of the activity against benzoyl-D-alanyl-thioglycolic

acid (S2d) as a function of the time are shown in the same table.

Intrinsic penicillin resistance phenomena are generally attributed to a decrease of 'affinity' of one or several PBPs for β-lactams. In some cases, it has been demonstrated that a modification of the amounts of the PBPs also contributed to these phenomena [11,23,24].

According to the three step model:

$$E + C \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EC \xrightarrow{k_2} EC^* \xrightarrow{k_3} E + P(s)$$

a decrease of the PBP 'affinity' could be due to: (i) an increase of the dissociation constant $K = k_{-1}/k_{+1}$; (ii) a decrease of the first order rate constant k_2; (iii) an increase of the first order rate constant k_3.

The results reported here clearly demonstrated that with the various β-lactams, the k_3 parameter was not affected in the modified C604 enzyme and that in consequence the decrease of penicillin binding was only due to the decrease of the acylation efficiency characterized by k_2/K. It was not possible to separate the effects of the mutations on the individual values of k_2 and K.

The alteration of the PBP2x* sequence which decreased the efficiency of acylation by cefotaxime, also affected the interactions with the other β-lactams. The mutation generally affected cephalosporins more than penicillins. However, acylation by a few penicillins was also serious impeded and the largest effect was observed with oxacillin. A coherent structure–activity relationship cannot be established without a knowledge of the tertiary structure of the enzyme.

With other bacteria, a correlation could sometimes be established between the MIC values and the saturation of a specific PBP [24–27]. In *S. pneumoniae*, no such simple relationship could be observed. With some antibiotics, cell lysis could be grossly correlated with the partial inhibition of PBP2b [28] but the modifications of penicillin binding properties observed in transformants of various resistance levels clearly indicated a possible implication of several or of all the HMW PBPs in the increased resistance [16].

The comparison of the k_2/K for both PBP2x* with the MIC values for both sensitive and low level resistant strains showed that:

(1) The increased resistance of strain C604 vs. cefotaxime is mainly due to a decreased affinity of this PBP for cefotaxime. Moreover, if PBP2x can be safely identified as the lethal target in the R6 strain, the MIC value for C604 only exhibited a 30-fold increase vs. a 300-fold decrease of the PBP2x* k_2/K value. This indicates that this MIC value probably reflects the appearance of other PBPs as important targets in the mutant strain.

(2) With the other β-lactams, the situation appears to be more complex. The relative values of the k_2/K ratios and of the MICs might indicate PBP2x to be also the target or one of the major targets of benzylpenicillin, oxacillin and piperacillin in the R6 strain (Table II). If PBP2x is the main target of a group of antibiotics, one would expect that a more-or-less constant value should be obtained when multiplying the MIC for an antibiotic by the corresponding k_2/K value. Indeed, if the MIC is expressed in M, the product MIC × k_2/K is a first-order

Table I

MIC values for the penicillin-sensitive R6 and cefotaxime-resistant C604 strains and comparison of the kinetic parameters for the interactions between their PBP2x and various antibiotics

Antibiotics	R6			C604		
	k_2/K ($M^{-1} \cdot s^{-1}$)	k_3 ($\cdot 10^5\ s^{-1}$)	MIC ($\mu g \cdot ml^{-1}$)	k_2/K ($M^{-1} \cdot s^{-1}$)	k_3 ($\cdot 10^5\ s^{-1}$)	MIC ($\mu g \cdot ml^{-1}$)
Penicillin						
Benzylpenicillin	58,000 ± 5,000	5.5 ± 0.5	0.01	13,200 ± 3,000	5.3 ± 1	0.01
Carbenicillin	3,600 ± 100	1.5 ± 0.5	ND	740 ± 100	ND	ND
Ticarcillin	1,400 ± 200	2.9 ± 1.0	ND	87 ± 5	ND	ND
Temocillin	650 ± 80	ND	ND	25 ± 10	ND	ND
Oxacillin	21,000 ± 2,000	13 0 ± 2.0	0.08	11 ± 5	ND	0.08
Piperacillin	53,000 ± 3,000	0.9 ± 0 5	0.02–0 04	30,000 ± 2,000	ND	0 02–0.04
Methicillin	4,900	ND	ND	ND	ND	
Carbapenem						
Imipenem	107,000 ± 3,000	0.06 ± 0.02	<0.01	2,000 ± 100	ND	<0.01
Cephalosporin						
Cephalexine	1,600 ± 100	2.1 ± 0.5	8	13 ± 3	2.6 ± 1.0	8–16
Cephaloglycin	12,400 ± 3,000	4.8 ± 1.0	ND	84 ± 10	ND	ND
Cephaloridine	93,000 ± 3,000	0.18 ± 0.05	ND	2,200 ± 100	0 21 ± 0.05	ND
Cefotoxitine	700 ± 100	0.40 ± 0.10	<1	26 ± 8	0.50 ± 0.10	2
Cefotaxime	162,000 ± 4,000	0.10 ± 0.02	0.02	510 ± 30	0.17 ± 0.05	0.6
Nitrocefin	990,000 ± 40,000	0.46 ± 0.10	ND	5,900 ± 100	ND	ND

The MIC values obtained by serial 2-fold dilutions might thus be inaccurate by a factor of 2. ND = not determined

Table II

Relationships between the MIC values for the cells and the k_2/K parameters for PBP2x in the sensitive and resistant strains

Antibiotics	MIC × k_2/K (s^{-1})	
	R6	C604
Benzylpenicillin	0.0018	0.0004
Oxacillin	0.0042	$2.2 \cdot 10^{-6}$
Piperacillin	0.0018–0.0037	0.0010–0.0021
Cephalexin	0.0370	0.0003
Cefotaxime	0.0071	0 0007

rate constant characteristic of the rate of acylation of the free PBP2x at this antibiotic concentration. If the value of k_3 is not changed and the modified PBP2x remains the target in the mutant, the MIC × k_2/K product should be the same as that observed with the wild type. A lower value of this product indicates that another PBP must be the target of the antibiotic and thus, PBP2x is certainly *not* the lethal target of oxacillin in the C604 strain. Moreover the behaviour of cephalexin clearly indicates that other PBPs must be involved in determining the MIC value. In consequence, it seems safe to assume that more than one HMW PBP must be inactivated to kill the cells but not all of them. Under these conditions, a PBP might appear as the 'primary' target if its inactivation is the 'last straw' leading to cell death.

The results obtained here present the PBP2x as a model to study the influence of the mutations occurring in laboratory mutants and in clinical isolates of resistant strains of *S. pneumoniae* by directly measuring the interaction between the protein and the β-lactam compounds. It should provide a new and helpful tool for understanding the mechanisms responsible for intrinsic β-lactam resistance in this bacterial species.

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REFERENCES

[1] Laible, G., Keck, W., Lurz, R., Mottl, H., Frère, J.M., Jamin, M. and Hakenbeck, R. (1992) Eur. J. Biochem 207, 943–949.
[2] Frère, J.M. and Joris, B. (1985) CRC Crit. Rev. Microbiol. 11, 299–396.
[3] Frère, J.M., Nguyen-Distèche, M., Coyette, J. and Joris, B. (1992) in: The Chemistry of Betalactams (Page, M.I., Ed.) pp. 148–195, Chapman and Hall, Glasgow.
[4] Malouin, F. and Bryan, L.E. (1986) Antimicrob. Agents Chemother. 30, 1–5
[5] Boulnois, G.J. (1992) J. Gen. Microbiol. 138, 249–259.
[6] Hakenbeck, R., Tarpay, M. and Tomasz, A. (1980) Antimicrob. Agents Chemother. 17, 364–371.
[7] Zighelboim, S. and Tomasz, A (1980) Antimicrob. Agents Chemother. 17, 434–442.
[8] Hakenbeck, R., Laible, G., Briese, T., Martin, C., Chalkley, L., Latorre, C., Kallıokoski, R. and Leinonen, M. (1991) in: Genetics and Molecular Biology of Streptococci, Lactococci and Enterococci (Dummy, G.M., Cleary, P.P. and McKay, L.L., Eds.) pp. 92–95, American Society for Microbiology, Washington, DC.
[9] Hakenbeck, R., Briese, T. and Ellerbrok, H. (1986) Eur. J Biochem. 157, 101–106.
[10] Hakenbeck, R. and Kohiyama, M. (1982) Eur. J. Biochem. 127, 231–236.
[11] Laible, G and Hakenbeck, R. (1987) Mol. Microbiol. 1, 355–363.
[12] Laible, G., Spratt, B.G. and Hakenbeck, R. (1991) Mol. Microbiol. 5, 1993–2002.
[13] Zighelboim, S. and Tomasz, A. (1979) in: Micronbiology (Schlessinger, D., Ed.) pp. 290–292, American Society for Microbiology, Washington, DC
[14] Dowson, C G., Hutchison, A., Brannigan, J.A., George, R.C., Hansman, D., Linares, J., Tomasz, A., Smith, J.M. and Spratt, B.G. (1989) Proc. Natl Acad. Sci. USA 86, 8842–8846.
[15] Laible, G., Hakenbeck, R., Sicard, M.A., Joris, B and Ghuysen, J.M. (1989) Mol. Microbiol. 3, 1337–1348.
[16] Laible, G. and Hakenbeck, R. (1991) J. Bacteriol. 173, 6986–6990.
[17] Jamin, M., Damblon, C., Millier, S., Hakenbeck, R. and Frère, J M. (1993) Biochem. 292, 735–741.
[18] Matagne, A., Misselyn-Bauduin, A M., Joris, B., Erpicum, T., Granier, B. and Frère, J M. (1990) Biochem. J. 265, 131–146
[19] Adam, M., Damblon, C., Plaitin, B., Christiaens, L. and Frère, J M (1990) Biochem. J. 270, 525–529.
[20] Adam, M., Damblon, C., Jamin, M., Zorzi, W., Dusart, V., Galleni, M., el Kharroubi, A., Piras, G., Spratt, B.G., Keck, W., Coyette, J., Ghuysen, J.M., Nguyen-Distèche, M. and Frère, J.M. (1991) Biochem. J. 279, 601–604.
[21] Wilkin, J.M., Jamin, M., Damblon, C., Zhao, G., Joris, B., Duez, C. and Frère, J.M. (1993) Biochem. J. 291, 537–544.
[22] Wilkin, J M., Jamin, M., Joris, B. and Frère, J.M. (1993) Biochem. J. 292, in press.
[23] Giles, A.F. and Reynolds, P.E. (1979) Nature 280, 167–168; Hakenbeck, R., Tarpay, M. and Tomasz, A (1980) Antimicrob. Agents Chemother 17, 364–371.
[24] Fontana, R., Cerini, R., Longoni, P., Grossato, A. and Canepari, P. (1983) J. Bacteriol. 155, 1343–1350.
[25] Spratt, B.G. (1977) Eur J. Biochem. 72, 341 352.
[26] Chase, H.A., Reynolds, P.E. and Ward, J.B. (1978) Eur J. Biochem. 88, 275–285.
[27] Reynolds, P.E., Shepherd, S.T. and Chase, H A. (1978) Nature 271, 568–570.
[28] Williamson, R., Hakenbeck, R and Tomasz, A. (1980) Antimicrob. Agents Chemother. 18, 629–637.