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The kinetic properties of the carboxy terminal domain of the Bacillus licheniformis 749/l BlaR penicillin-receptor shed a new light on the derepression of β-lactamase synthesis

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Summary

To study the properties of the BlaR penicillin-receptor involved in the induction of the *Bacillus licheniformis* β-lactamase, the water-soluble carboxy terminal domain of the protein (BlaR-CTD) was overproduced in the periplasm of *Escherichia coli* JM105 and purified to protein homogeneity. Its interactions with various β-lactam antibiotics were studied. The second-order acylation rate constants K_2/K' ranged from 0.0017 to more than 1 $\mu M^{-1} s^{-1}$ and the deacylation rate constants were lower than 4×10^{-5} s^{-1}. These values imply a rapid to very rapid formation of a stable acylated adduct. BlaR-CTD is thus one of the most sensitive penicillin-binding proteins presently described. In the light of these results, the kinetics of β-lactamase induction in *Bacillus licheniformis* were re-examined. When starting with a rather high cell density, a good β-lactamase substrate such as benzylpenicillin is too sensitive to β-lactamase-mediated hydrolysis to allow full induction. By contrast, a poor β-lactamase substrate (7-aminocephalosporanic acid) can fully derepress β-lactamase expression under conditions where interference of the antibiotic with cell growth is observed. These results suggest that acylation of the penicillin receptor is a necessary, but not sufficient, condition for full induction.

Introduction

The *Bacillus licheniformis* BlaP and *Staphylococcus aureus* BlaZ class A β-lactamases are inducible by β-lactam antibiotics (Joris *et. al,* 1994; Philippon *et al.,* 1998). In both strains, the regulation of β-lactamase production involves three regulatory genes, *blal, blaR1* and *blaR2.* The first two genes encode, a repressor and a penicillin sensory transducer respectively, whereas the third is not yet identified (Sherrat and Collins, 1973; Dyke, 1979; Kobayashi *et al.,* 1987; Nicholls and Lampen, 1987; Rowland and Dyke, 1990). In *Bacillus licheniformis* 749/I, *blal* and *blaR1* are located on the chromosome downstream the structural *blaP* gene and are transcribed in the opposite direction. The additional *blaR2* gene has been highlighted by genetic analysis and is not linked to the *blaP-blal-blaR1* locus. Some *blal*-mutants give rise to magnoconstitutive expression of the BlaP β-lactamase, and *blaR1*-mutants are non-inducible and have normal levels of β-lactamase in the absence of inducer. Some *blaR2*-mutants exhibit about half the basal β-lactamase activity of the wild-type and do not show a significant induction with penicillin.

The membrane topology of *B. licheniformis* BlaR (601 amino acids) has been determined experimentally (Hardt *et al.,* 1997). The amino-terminal domain of the protein (BlaR-NTD, Met^{1}-Pro^{339}) exhibits four transmembrane segments. A zinc metalloprotease motif is present within the large cytoplasmic domain (Tyr^{134}-Arg^{322}). Its carboxy-terminal counterpart (BlaR-CTD, Ser^{340}-Arg^{601}) contains the motifs of the penicilloyl serine transferases and protrudes from the external face of the cytoplasmic membrane. This sensor domain is highly isologous to class D β-lactamases (Zhu *et al.,* 1990). After production as a water-soluble protein in the periplasm of *Escherichia coli,* BlaR-CTD was characterized as a penicillin-binding protein and as a member of the active-site serine penicillin-recognizing enzyme family (Joris *et al.,* 1990). The two models which have been proposed to explain the induction of BlaP/BlaZ β-lactamases differ at the level of the proteolytic mechanisms responsible for the inactivation of the Blal repressor. In *S. aureus,* Zhang *et al.* (2001) proposed a direct inactivation of Blal whereas Filée *et al.* (2002) proposed that Blal inactivation was mediated by a co-activator (for details see Fig. 1).

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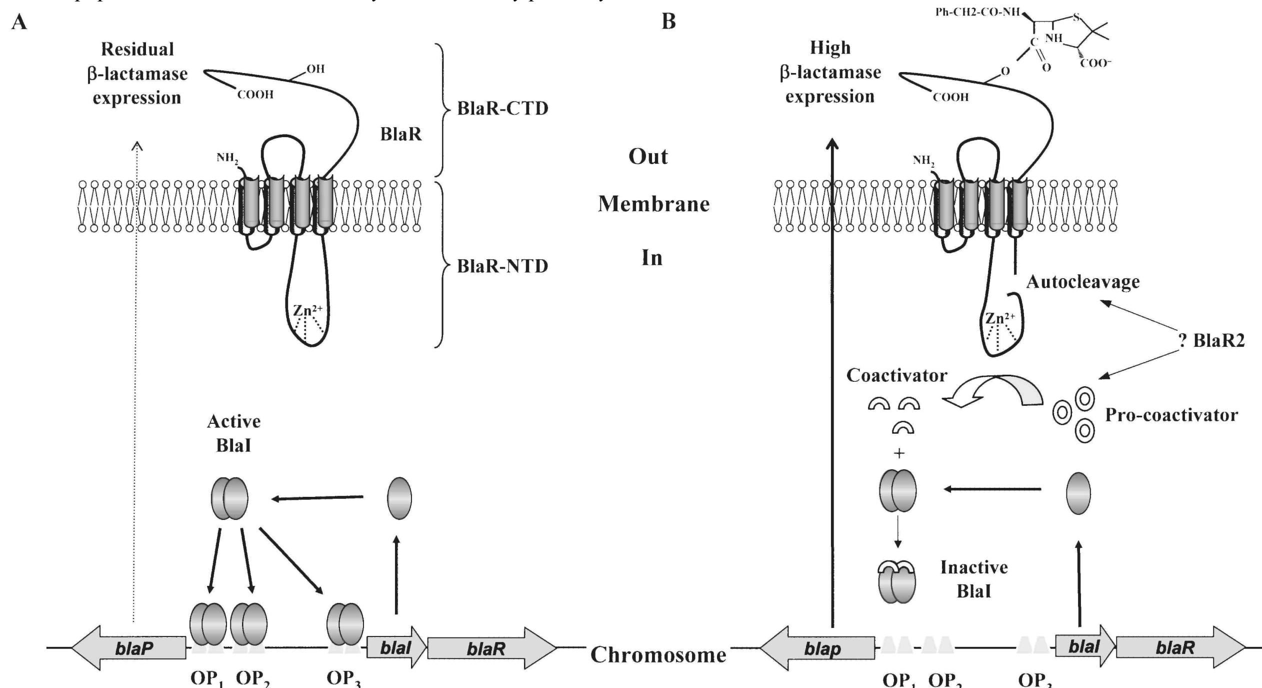
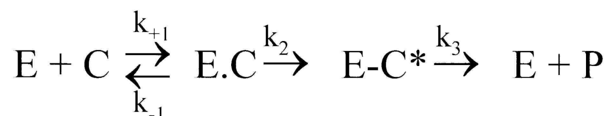
Fig. 1. *β-lactamase induction mechanism in B. licheniformis 749/I.*
A. In the absence of penicillin, transcription of the blaI gene results in synthesis of the BlaI repressor whose dimer binds to three operators (OP1, OP2 and OP3) located in the intergenic region between blaP and the blaI-blaR operon and prevents the transcription of both the structural and the regulatory genes at a high level.
B. When a β-lactam antibiotic is added in the medium, the extracellular penicillin-binding domain of BlaR (BlaR-CTD) is acylated and a signal passes throught the membrane causing the proteolytic activation of the intracellular metallopeptidase domain (Zhang et al. 2001; K. Benlafya, unpubl. data). In B. licheniformis, it has been postulated that the activated metallopeptidase converts a pro-co-activator into a co-activator which binds to the BlaI repressor and displaces it from its DNA-binding sites (Filée et al. 2002). According to Zhang et al. (2001) in S. aureus, BlaI itself would be the substrate of the metallopeptidase and would be directly inactivated by proteolysis.



The interaction between the active-site serine penicillin recognizing enzymes and β-lactams involves the formation of an acyl-enzyme intermediate where the antibiotic is covalently bound to the active-site serine. The kinetic analysis demonstrated that the interaction obeyed a three-step model (Frère, 1995) (Fig. 2).

Fig. 2. *Interaction between an active-site serine penicillin-recognising enzyme and a β-lactam antibiotic. E is the enzyme, C the β-lactam, E.C the Henri-Michaelis complex, E-C* the acyl-enzyme and P the product of hydrolysis.*



In contrast with serine β-lactamases, PBPs do not efficiently catalyse the deacylation step and the acyl-enzyme can be easily isolated. In the general case, the pseudo-first order rate constant for acylation (k_a) is given by

$$k_3 + \frac{k_2 . k_{+1} . [C]}{(k_{-1} + k_2) + [C]} \text{ or } k_3 + \frac{k_2 . [C]}{K'} \text{ where } K' = \frac{k_{-1} + k_2}{k_{+1}}$$

With PBPs, k_3 is usually negligible and k_2 much smaller than k_{-1}, so that

$$k_a = \frac{k_2 . [C]}{K + [C]} \text{ where } K = \frac{k_{-1}}{k_{+1}}$$

i.e the dissociation constant of EC. Most often, high acy-lation rates are observed at [C] < < K so that the important kinetic parameter is K_2/K. K_3 is determined in separate experiments (see *Experimental procedures).* With BlaR however, k_a/[C] is so high that the assumption K'≈K might not be true. In the text, K' will thus be

used for BlaR and K for other PBPs. With β-lactamase, the usual steady-state equations apply and, unless contradictory evidence has been obtained,

$$k_{cat} = \frac{k_2.k_3}{k_2 + k_3} \text{ and } K_m = \frac{k_3.K}{k_2 + k_3}.$$

In this paper, we report the analysis of the kinetics of interaction between BlaR-CTD and a set of β-lactams antibiotics and we show that the protein is very sensitive to all the tested compounds. The impact of these properties on β-lactamase induction in *B. licheniformis* 749/I were analysed with a good (benzylpenicillin) and a poor (7-aminocephalosporanic acid, 7-ACA) β-lactamase substrates.

Results and discussion

Production and purification of BlaR-CTD

After transformation by pDML310, *E. coli* JM105 produced and exported into the periplasm a polypeptide identical to that produced by the same strain harbouring pDML307 (Joris *et al.,* 1990). The production of BlaR-CTD by *E. coli* strain JM105/pDML310 was estimated to 15 mg per litre of culture, i.e. 15 times larger than that obtained previously with *E. coli* JM105/pDML307.

Table 1 summarizes the purification of BlaR-CTD from a 15 litre culture. BlaR-CTD was at least 99% pure as estimated by SDS-PAGE. Active-site titration was performed with [^{14}C] benzylpenicillin and complete acylation occurred at an inactivator/enzyme ration of 1.1 - 0.1.

Table 1. *Purification of BlaR-CTD.*

	Total proteins (mg)	Purity (%)	Recovered BlaR-CTD (mg)
Crude periplasmic extract	4500	5	225
Q Sepharose Fast Flow	198	80	158 (70)
Phenylboronic acid Sepharose	142	>99	142 (90)

The yield (%) of each step is given between parentheses.

Kinetic measurements

Table 2 lists the kinetic parameters determined on a representative set of β-lactam antibiotics. These values reflect an exceptionally high sensitivity of BlaR-CTD to most studied β-lactams. For instance, the rate of acylation by benzylpenicillin is more than 20-fold higher than that observed with the *Actinomadura* R39 DD-peptidase (Fuad *et al.,* 1976) and *Bacillus licheniformis* PBP1 (Lepage *et al.,* 1995a) which are usually considered as extremely sensitive to β-lactams. The acylation rate by ampicillin (1.3 $\mu M^{-1} s^{-1}$) is very close to the k_{cat}/K_m value of the OXA-2 β-lactamase for the same compound (Ledent *et al.,* 1993). In consequence the rate of acylation by ^{14}C-benzylpenicillin was measured by quenched-flow and at an antibiotic/protein molar ratio of 1, thus under second-order kinetics conditions, where the reaction half-time was about 60 ms. BlaR-CTD is not the most sensitive PBP for *all* β-lactams (for instance, the R39 peptidase is somewhat more sensitive to cephal-exin and cephaloglycin) (Fuad *et al.,* 1976) and *Streptococcus pneumoniae* PBP2x more sensitive to cefotaxime (Jamin *et al.,* 1993). In all cases, the k_3 values were low to very low. It is clear that compounds devoid of side-chain (6-APA and 7-ACA) exhibit low relative acylation rates (when compared to the best compounds) as observed with all PBPs. Large side-chains or a penam nucleus are clearly unfavourable (methicillin, cloxacillin). With the exception of cephalori-din, cephalosporins are generally less efficient. If one excepts 7-ACA, the poorest inactivators are cephalexin (a cephalosporin devoid of leaving group), the monobactam aztreonam and mecillinam, an amidinopenicillin. At the present time, it has proved very difficult to explain the variations of acylation rate constants on the basis of the structure of PBPs and β-lactams and this will not attempted here.

Table 2. *Acylation rate constants (k_2/K) and deacylation rate constants (k_3) of BlaR-CTD with various β-lactams.*

β-lactam antibiotic	k_2/K (μM^{-1}s^{-1})	k_3 (s^{-1})
Penicillins		
Ampicillin	1.3 ± 0.1	<10^{-5}
Amoxycillin	1.3 ± 0.3	<10^{-5}
Carbenicillin	0.55 ± 0.02	1.6 10^{-5}
Methycillin	0.26 ± 0.02	1.4 10^{-4}
Cloxacillin	0.19 ± 0.02	3 10^{-5}
6 APA	0.06 ± 0.02	ND
Benzylpenicillin	8.7 ± 1.1	1.4 10^{-5}
Cephalosporins		
Cephalexin	1.7 10^{-3} ± 0.2 10^{-3}	ND
Cephaloglycin	0.041 ± 0.004	<10^{-5}
Cephaloridine	5.9 ± 0.2	<10^{-5}
Cefotaxime	0.043 ± 0.003	2.7 10^{-5}
Cefuroxime	0.022 ± 0.005	1.1 10^{-5}
Cefthiofur	0.1 ± 0.03	3.5 10^{-5}
Cephapirin	0.52 ± 0.07	<10^{-5}
7-ACA	1.7 10^{-3} ± 0.3 10^{-3}	<10^{-5}
Others		
Cefoxitine	0.064 ± 0.02	<10^{-5}
Moxalactam	0.36 ± 0.04	<10^{-5}
Aztreonam	3.6 10^{-3} ± 1 10^{-3}	ND
Mecillinam	4.8 10^{-3} ±0.3 10^{-3}	ND

It seems very likely that the rate constants measured here for BlaR-CTD can be extrapolated to the complete, membrane-bound protein. Indeed, the structural analysis presented before (Joris *et al.,* 1990) suggests that the C-terminal part of the protein behaves as a globular, independent domain exposed on the external face of the cyto-plasmic membrane. Essentially, the present data suggest that acylation of BlaR would be more than 90% complete within one min at a 1 μM concentration of all the compounds exhibiting a k_2/K-value larger than 0.05 μM^{-1} s^{-1}.

This acylation represents the first step of signal transmission, but does not appear to be sufficient to allow complete derepression of β-lactamase synthesis. The problem is complicated by the fact that many β-lactams are sensitive to the *B. licheniformis* β-lactamase, so that the initial concentration of inducer is not maintained throughout the period of time necessary to reach full induction. To further explore the relationship between BlaR acylation and derepression of the β-lactamase synthesis, induction experiments were performed with a very good (benzylpenicillin) and a very poor (7-aminocephalosporanic acid) β-lactamase substrates.

Induction by benzylpenicillin

Figure 3 shows the effects of the addition of various concentrations of benzylpenicillin to a *B. licheniformis* 749/I culture which had reached an A^{550} value of 0.1. An increase of the benzylpenicillin concentration influences the growth rate (Fig. 1A). During the first 30 min of induction, the variation of A^{550} is 0.0072 min^{-1} and 0.0079 min^{-1} in the absence and presence of 50 nm of benzylpencillin, respectively, whereas this variation decrease at 0.0042 A^{550} min^{1} and 0.0022 A^{550} min^{-1} with 1 μM and 5 μM benzylpenicillin. Under all induction conditions, the growth rate reaches a maximum (0.015-0.018 A^{550} min^{-1}) and decreases after 200 min of β-lactamase induction. The amount of secreted β-lactamase produced never exceeded 350 μg l^{-1}. A^{550}, i.e. 15- to 25-fold the basal level. Note that, before addition of the inducer an equal amount of cell-bound β-lactamase is observed. In presence of inducer, this proportion thereafter rapidly decreases to about 20% when A^{550} = 0.2. With a low benzylpenicillin concentration (50 nM), β-lactamase production is slow during the first 30 min (0.47 μg l^{-1}.A^{550}.min) and little β-lactamase is produced at the beginning of induction. This production increases afterwards to reach the highest maximum rate of production (2.57 μg l^{-1}.A^{550}.min). With higher benzylpenicillin concentrations (1 μM and 5 μM), the rate of β-lactamase production is initially higher than that obtained with 50 nM benzylpenicillin (0.67 and 1.04 μg l^{-1}.A^{550}.min, respectively, during the first 30 min) but, subsequently, never reaches the level obtained with 50 nM benzylpenicillin (Fig. 3C). The maximum rates of β-lactamase production with 1 μM and 5 μM benzylpenicillin are 1.91 and 1.04μg l^{-1}.A^{550}.min respectively (Fig. 3D). To underline the possible importance of the time of contact between the antibiotic and the cells, benzylpenicillin was destroyed after increasing periods of time by the addition of the *B. cereus* metallo-β-lactamase (see *Experimental procedures).* The results are depicted by Fig. 4. The chosen benzylpenicillin concentration (20 nm) does not affect bacterial growth (Fig. 4A)

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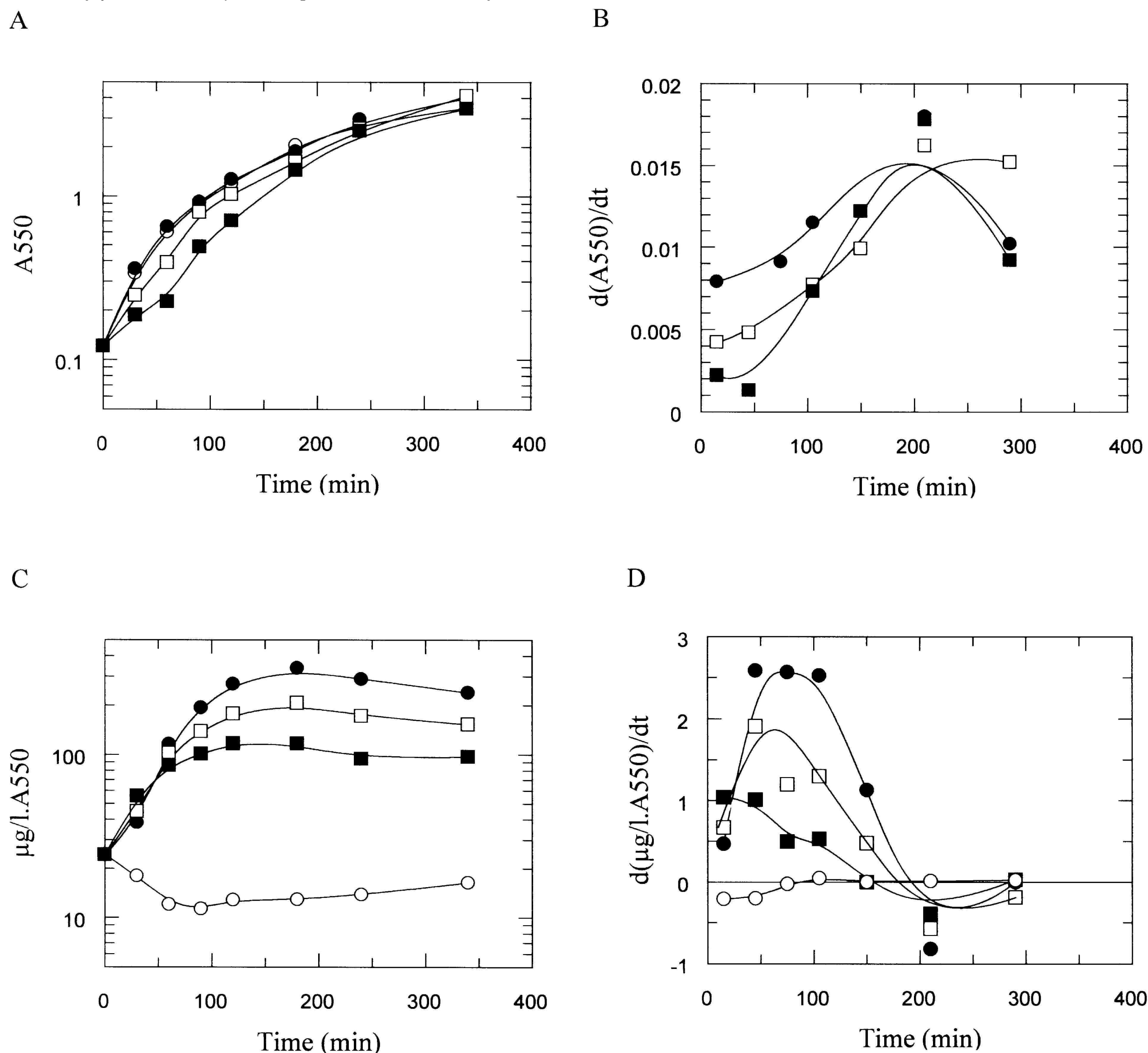
but is sufficient to rapidly acylate BlaR ($t_{1/2}$ = 4-5 s). When benzylpenicillin was destroyed after 5 min, β-lactamase production was barely above the non-induced level.

Fig. 3. *Effect of benzylpenicillin on the growth of B. licheniformis 749/I and on β-lactamase induction. The antibiotic was added when the A550 of the culture reached a value of 0.1 (time 0).*
A. Comparison of the growth curves without benzylpenicillin (non-induced ○) or with 50 nM (●), 1 μM (□), 5 μM (■) antibiotic.
B. Growth rate [derivative of (A)].
C. Specific activity of the induced β-lactamase.
D. Rate of β-lactamase synthesis per cell [derivative of (C)].



Thereafter the β-lactamase production became more significant and, in a first approximation, proportional to the time of contact, but did not further increase after this 'time of contact' reached 30 min. There is however, a rather simple explanation for this phenomenon. With the basal level of β-lactamase produced (2-8 μg l^{-1} during the first 20 min), and on the basis of the kinetic parameters determined before (Matagne *et al.,* 1990), the half-life of the antibiotic would be expected to be in the range of 1.2-6 min and the residual concentration after 30 min would be negligible (Table 3). Figure 4 also shows that the rate of β-lactamase synthesis per cell (Fig. 4D) becomes negligible when the growth rate reaches its maximum (Fig. 4B).

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Fig. 4. *Induction of the B. licheniformis 749/I β-lactamase by benzylpenicillin.*
A. Growth curves in the absence (○) and the presence of 20 nM benzylpenicillin (∇).
B. Growth rate (derivative of A).
C. β-lactamase production. Residual benzylpenicillin was destroyed after 5 (●), 15 (□), 20 (■), 30 (Δ) and 40 min (▲). The other symbols are as in (A).
D. Rate of β-lactamase synthesis per cell [derivative of (C)].

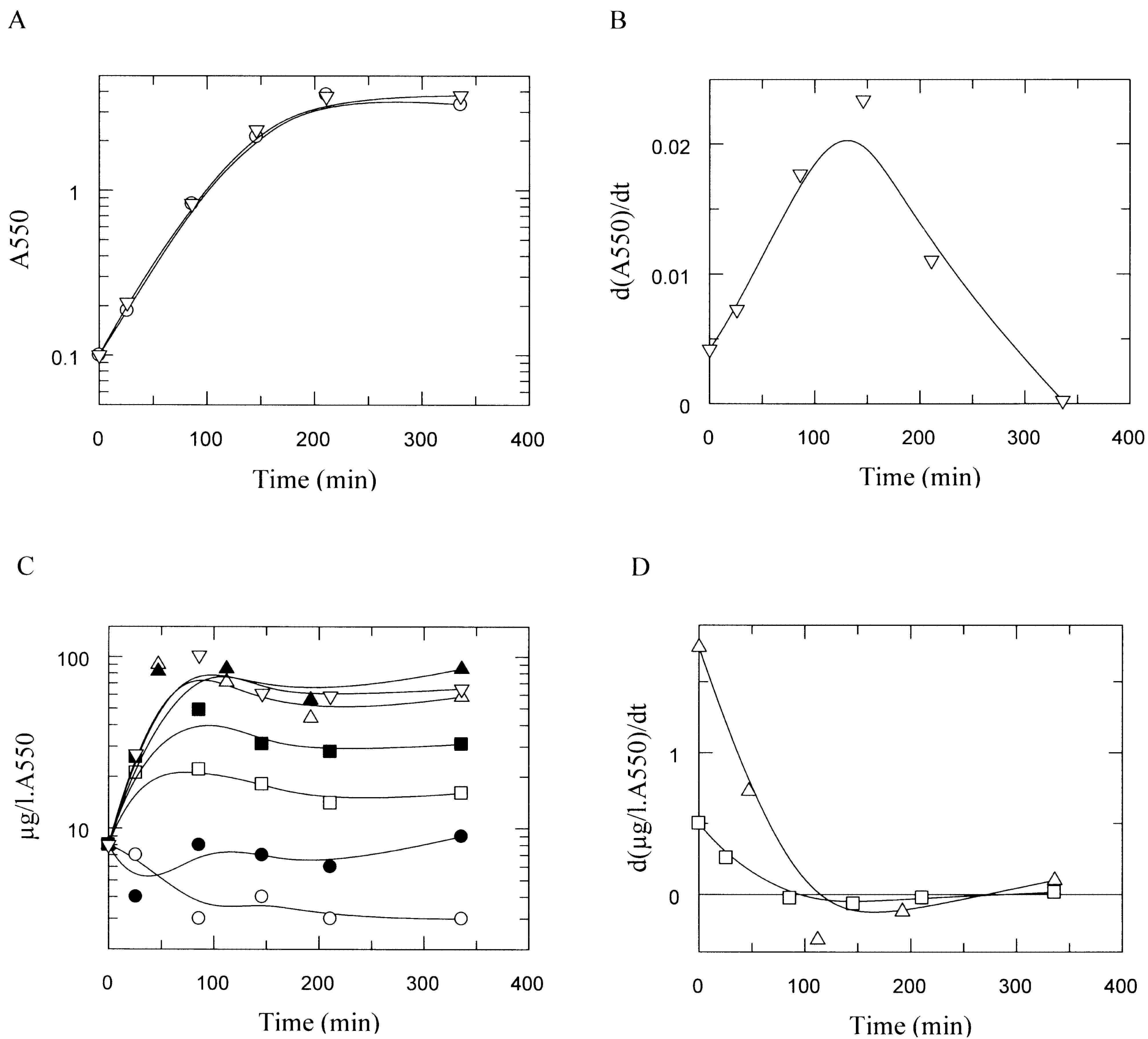


Table 3. *Time needed to reduce the benzylpenicillin concentration to 20 nM (t_{20}) or 5 nM (t_5) with a β-lactamase concentration of 4 µg l^{-1}. Note that the MIC of a B. licheniformis 749 mutant which produces an inactive β-lactamase is 20 nM (see text).*

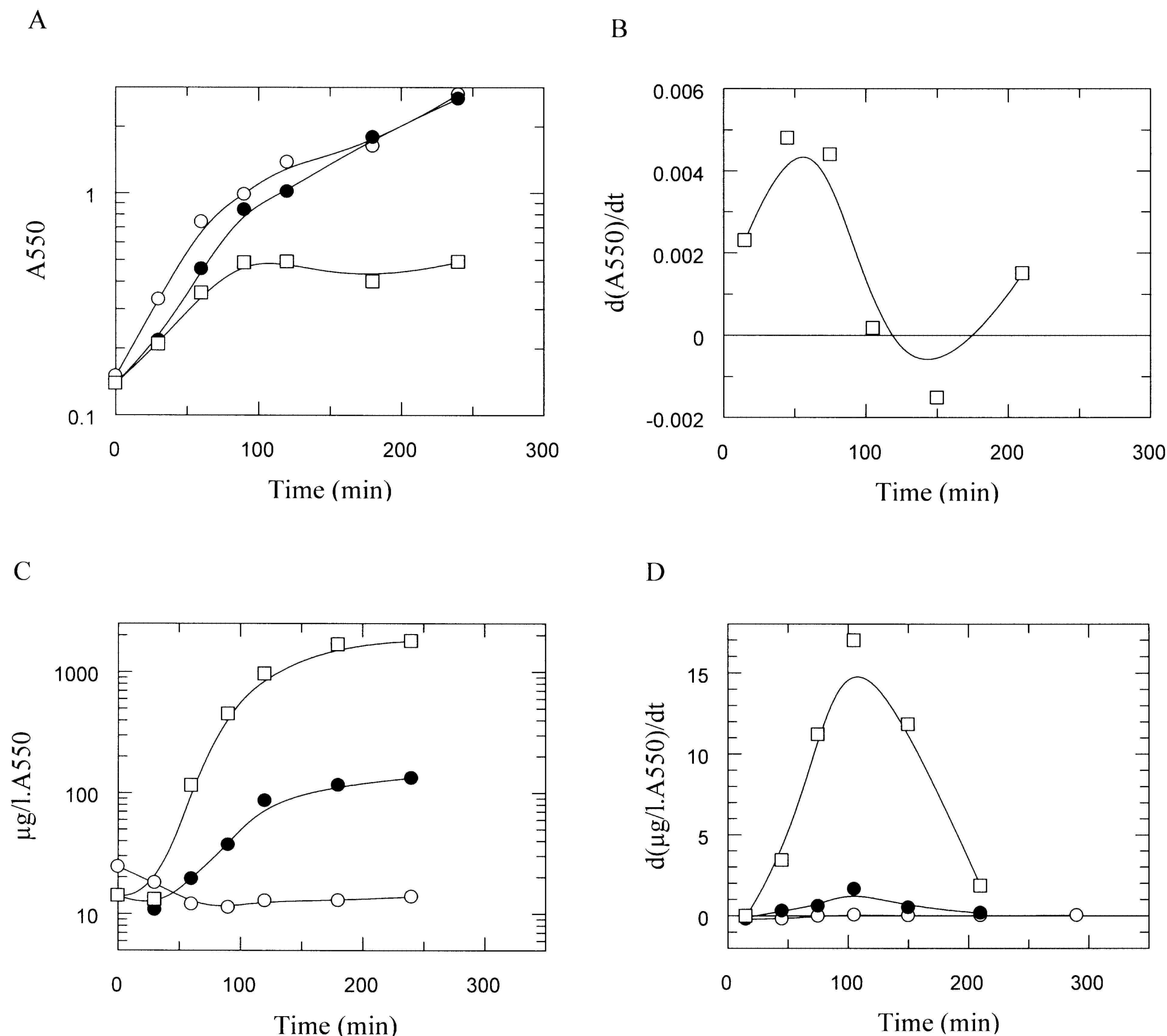
Initial penicillin concentration (µM)	t_{20} (min)	t_5 (min)
0.3	11	17
0.9	16	22
2	19	25
4	22	28
10	26	32

Induction by 7-aminocephalosporanic acid (7-ACA)

Figure 5 depicts similar experiments performed with 7-aminocephalosporanic acid (7-ACA). With 2µM 7-ACA, the variation of A^{550} is first 0.0026 min^{1} and reaches a maximum (0.0128 min^{1}) as in the experiments performed with benzylpeniccilin. With 18.5 µM 7ACA, the growth rate is more affected (Fig. 5A and B). The variation of A^{550} is first 0.0023 min^{-1}. This value increases only to 0.0048 min^{-1} and rapidly decrease thereafter to zero. On the other hand, here, a much higher production level of β-lactamase per cell is reached and it is quite clear that maximum induction (1780 µg l^{-1}. A^{550}) is obtained with antibiotic concentrations which significantly affect the growth rate (Fig. 5C and D). The rate of β-lactamase synthesis per cell is maximum (maximum rate of production = 17) when the growth rate is minimum (compare Fig. 5B and D) and decreases when growth slowly resumes 200 min after addition of the inducer.

On the basis of the kinetic parameters for the hydrolysis of 7-ACA by the *B. licheniformis* β-lactamase (IW K_m = 330 M^{-1} s^{-1}, K_m = 220 µM), no significant hydrolysis of the antibiotic is expected over a 4 h period, even at an enzyme concentration of 1000 µg l^{-1}, which is the highest one measured after 180 min of induction with 18.5 µM 7-ACA (taking account of the cell-bound enzyme). Under these conditions, BlaR is rapidly acylated (half-reaction time with 20 µM 7-ACA: 20 s) and remains so throughout the whole experiment. This is the major difference between the two β-lactams. Since the activation of BlaR derepresses its own synthesis (Salerno and Lampen, 1988) the system enters an autocatalytic cycle if the inducer remains present. In the case of benzylpenicillin, the cycle is interrupted by the disappearance of the antibiotic and the dilution of the acylated BlaR by newly synthesised intact molecules. Thus, the cycle does not reach full efficiency, so that β-lactamase production never reaches a maximum and the rate of enzyme synthesis per cell decreases to zero after about 100 min (Fig. 2D). (It should be noted, however, that the situation would be different if the same inducer were added at a much lower cell density than that utilized here.)

Fig. 5. *Effect of 7 aminocephalosporanic acid (7ACA) on the growth of B. licheniformis 749/I and β-lactamase induction. The antibiotic was added when the A^{550} of the culture reached a value of 0.1 (time = 0).*
A. Comparison of the growth curves without (non-induced ○) or with 2 μM (●) and 18.5 μM (□) of 7ACA.
B. Growth rate [derivative of (A)].
C. Specific activity of the induced β-lactamase.
D. Rate of β-lactamase synthesis per cell [derivative of (C)].



Northern blotting analysis

Total mRNAs were prepared from cultures of wild-type *B. licheniformis* 749/I which were sampled during induction as described in *Experimental procedures.* Total mRNAs samples were submitted to Northern blotting analysis by hybridization with a biotinylated antisense *blaP* RNA probe.

When 50 nM and 1 μM benzylpenicillin was used for β-lactamase induction, the transcription of the 0.9 kbp *blaP* mRNA was induced in both conditions. The amount of this transcript reaches its maximum level after 60 min of induction and decreases after 90 min (Fig. 6A and B).

When 2 μM 7-ACA was used for β-lactamase induction, the transcription of the *blaP* mRNA was slowly and continuously induced (Fig. 6C). With 18.5 μM 7-ACA, the maximum amount of transcript is obtained after 30 min of induction and never decreases during the experiment (Fig. 6D). These results are in agreement with those obtained by following the β-lactamase expression.

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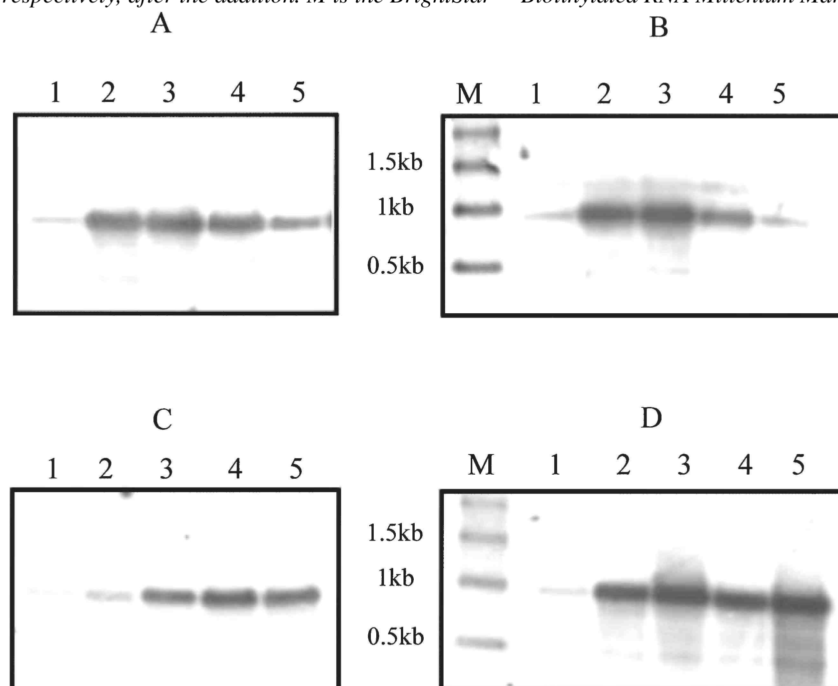
Fig. 6. *Induction of transcription of the blaP gene in B. licheniformis 749/I by 50 nM (A) and 1 µM (B) benzylpenicillin and 2 µM (C) and 18.5 µM (D) 7-aminocephalosporanic acid (7ACA). Northern analysis of total RNA (0.75 µg) prepared from cell grown at 37°C before adding antibiotic (lane 1) or 30 min (lane 2), 60 min (lane 3), 90 min (lane 4) and 120 min (lane 5), respectively, after the addition. M is the BrightStar™ Biotinylated RNA Millenium Markers™.*



Growth rate and PBP/acylation

But it does not seem that a complete acylation of BlaR during an adequately long period is sufficient to result in full induction. Indeed, with 2 µM 7-ACA, the half-reaction time for the acylation of BlaR is 200 s and simulations show that more than 90% of the receptor will be permanently acylated and thus activated. However, as seen in Fig. 5C, induction remains well below 10% of the maximum under these conditions. In consequence, it seems that another factor must be involved in the phenomenon. It has been noted above that the 7-ACA concentrations which result in the highest induction also significantly influence the growth rate. Earlier results (Lepage *et al.,* 1995a) have shown that PBP1 was the lethal target of benzylpenicillin and 7-ACA in *B. licheniformis* and, in the following discussion and in the simulations, we consider that inactivation of a large proportion of PBP1 is necessary to result in cell death. The second-order rate constant for the acylation of *B. licheniformis* PBP1 by 7-ACA (45 M^{-1} s^{-1}) has been measured before (Lepage *et al.,* 1995a). Thus, at a 20 µM concentration of 7-ACA, the half-reaction time for acylation of PBP1 is about 10 min. During the first hour after addition of 20 µM 7-ACA, growth continues at about 60% of the rate of the control culture. The simulations based on the assumption that the rate of PBP1 synthesis directly reflects the growth rate show that the proportion of free active PBP1 decreases to 50% after 18 min and stabilises around 32-33% after 50 min. Between the first and second hour, there is very little growth and β-lactamase induction becomes significant at the end of the second hour and further increases during the third hour. The level of PBP1 acylation after one hour is probably sufficiently high to account for the strongly decreased growth rate during the second hour. If the rate of PBP1 synthesis accordingly decreases, the proportion of acylated PBP1 then rapidly increases above 70%. By contrast, with 2 µM 7-ACA, less than 20% of PBP1 is acylated and growth is not impeded. This analysis clearly suggests that PBP1 acylation results in growth impairment and that the latter is a necessary condition for β-lactamase induction, thus establishing a correlation between pep-tidoglycan metabolism and this latter phenomenon. Benzylpenicillin acylates PBP1 much faster than does 7-ACA (k_2/K= 100 000-300 000 M^{-1} s^{-1} versus 45 M^{-1} s^{-1} Lepage *et al.,* 1995a). However as underlined above, it is highly sensitive to the *B. licheniformis* β-lactamase and the hydrolysis of the benzylpenicillin-PBP1 adduct is rather rapid ($k_3 = 2 \times 10^{-3}$ s^{-1}). It is easy to calculate that with 1—10 µM benzylpenicillin, the proportion of acylated PBP1 increases to more than 95% in less than 2 min. However, wihin 40 min, the benzylpenicillin concentration decreases below 20 nM (the MIC of a β-lactamase negative mutant, Lepage *et al.,* 1995a) and growth resumes after a lag of 20-110 min when compared to the control curve (Fig. 4).

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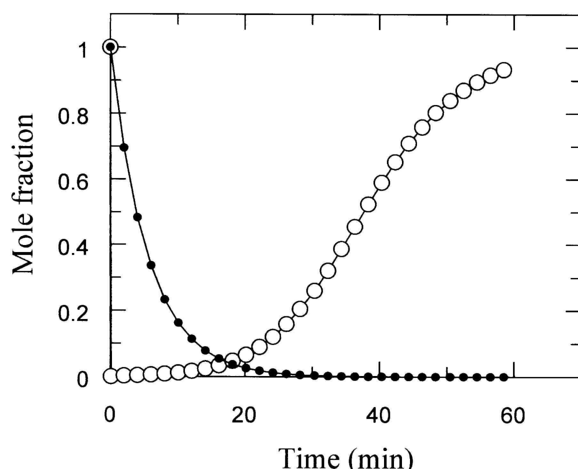
Figure 7 shows a simulation of the situation with 10 µM benzylpenicillin assuming that PBP1 synthesis is completely inhibited upon addition of the antibiotic. The proportion of free active PBP1 remains below 2% for about 12 min, reaches 50% after 36 min and 90% after 57 min. Over this period of time, some lysis occurs, but not enough to prevent the resumption of growth after 2 h. A large proportion of PBP1 (>50%) remains inactivated during more than one generation time, but this does not result in extensive lysis. Interestingly, after a nearly full recovery of PBP1 activity, it takes another 50-60 min before the culture starts growing again at a rate similar to that of the control. This leads to the conclusion that, to kill a very large proportion of the cells, permanent inactivation of PBP1 is necessary over more than one generation time. Similarly, the fact that with 5 µM benzylpenicillin, β-lactamase production never exceeds 10% of that observed with 7-ACA (Fig. 5) underlines a need for prolonged inactivation of PBP1 to reach full induction. Interestingly, cephalexin reacts with its target PBP (PBP3) faster than with BlaR, the *k_2/K-values* being 23000 (Lepage *et al.,* 1995b) and 1700 M^{-1} s^{-1} (Table 1) respectively. When cephalexin was added to a growing *B. licheniformis* 749/I as above, growth continued without induction (up to 0.3 µM cephalexin) and at a higher concentration (1 µM) complete lysis occurred rapidly.

Fig. 7. *Simulations of the disappearance of benzylpenicillin (●) and the ratio of acylated/free PBP1 (○) on the basis of the model presented in the Experimental procedures section. The following conditions were used: initial [benzylpenicillin] = 10 µM, initial [PBP1] = 0.01 µM (Lepage et al., 1995a), no synthesis of new PBP1 during the time-course, k_f = 100 000 $M^{-1}s^{-1}$, k_3 = 0.002 s^{-1} (Lepage et al., 1995a) and k_d = 0.03 s^{-1} (4 µg β-lactamase l^{-1}). Note that modification of the conditions in the direction of residual PBP1 synthesis or of an increased amount of β-lactamase would shorten the time needed for PBP1 recovery.*



Conclusions

On the basis of the data presented and discussed above, it appears that full derepression of β-lactamase synthesis by 7-ACA results from acylation of the BlaR detector necessarily accompanied by a strongly affected growth, which probably reflects an interference of the inducer with peptidoglycan metabolism. These results obtained with 7-ACA suggest that permanent inactivation of 70% of PBP1 over probably at least two generation times fulfils this latter condition. BlaR should similarly remain acylated (and thus, in this case, activated) for a sufficient time, but this is not a problem since its *k_2/K-values* seem to be larger than those of PBP1 (compare Table 2 with the data of Lepage *et al.*, 1995a) and the corresponding k_3 values are very small. The same conclusions can be deduced when benzylpenicillin is used as inducer. Finally two observations remain unexplained but do not affect our conclusion relating peptidoglycan metabolism to induction:

(1) Why does growth resume 200 min after addition of 18.5 µM 7-ACA?
(2) How can cells survive when PBP1 is fully acylated by benzylpenicillin during at least one generation time?

The first paradox might be explained by a slow disappearance of 7-ACA due to chemical hydrolysis and the second by a drastic modification of metabolism due to the stress conditions, modification which would allow some of the cells to survive until the β-lactamase has eliminated the PBP inactivator.

Experimental procedures

Sources of β-lactam antibiotics

The antibiotics used in this study were chosen as representatives of the various classes of β-lactam βantibiotics. Benzylpenicillin was from Rhône Poulenc, cefuroxime from Glaxo Group Research, cefotaxime from Hoeschst-Roussel, moxa-lactam from Merck, Sharp and Dohme Research Laboratories, cephaloridine, cephalexin and cephaloglycin from Eli Lilly. Ampicillin and cephapirin from Bristol Benelux, carbenicillin, cloxacillin, methicillin, amoxycillin and 6-aminopenicillanic acid (6-APA) from Beecham Research Laboratories, aztreonam from the Squibb Institute for Medical Research, cefthiofur from Upjohn and mecillinam from Leo Pharma. All these compounds were kindly given by the respective companies.

[^{14}C]Benzylpenicillin (50 mCi mmoL^{-1}) was purchased from Amersham Pharmacia Biotech, 7-aminocephalosporanic acid (7-ACA) from Acros Organics and nitrocefin from Oxoid.

Plasmids and bacterial strain

Plasmid pRTW8 (Kobayashi *et al.,* 1987) was the source of the *blaR* gene, plasmid pBGS18 + (Spratt *et al.,* 1986) was the source of the *Km*^{R} gene conferring resistance to kanamycin and plasmid pIN III-*ompA* (Rentier-Delrue *et al.,* 1988) was used as expression vector. This latter plasmid contains the Amp^R gene encoding resistance to ampicillin, the strong promoter, *lpp^P* of the *E. coli* lipoprotein, which is controlled by the /ac-UV5 promoter-operator, the *E. coli* OmpA signal peptide under the control of the two latter promoters and a polylinker with a *Hin*dlll recognition site just at the end of the OmpA signal peptide sequence. *Escherichia coli* JM105 (Yanisch-Perron *et al.,* 1985) [*lac*$^-$, *proAB, thi, rps*L, *end*A, *sbc*B15, *hsp*R4 (F', *pro*$^+$, B$^+$, *l*acl^{q}ZM15, *tra*D)] was used as host.

Genetic constructions

Standard recombinant techniques (Maniatis *et al.,* 1982) and sequencing techniques (Sanger *et al.,* 1977) were used.

After annealing of the two complementary 36-mer phosphorylated oligonucleotides containing internal *Smal, Sad* and Hin*d///* restriction sites, 5'-AGCTCAAGAAGCCACGTCCCGGGGAGCTCAAGCTTG-3' and 5'-AATTCAAGCTTGAGCTCCCCGGGACGTGGCTTCTTG-3', the resulting duplex provides cohesive *Hin*dlll and EcoRI specific ends allowing its cloning in the *Hin*dlll, EcoRI-digested plN III-*omp*A plasmid. The isolated and purified recombinant plasmid was called pDML308. In the next step the *bla*R-CTD gene, which extends from *Bal*l to *Hin*dlll in pRTW8, was cloned into the Smal and *Hin*dlll digested pDML308 in which the *Hin*dlll, *Smal* and *Sacl* restriction sites present in the polylinker are unique. In the next step the *blaR-CTD* gene, which extends from *Ball* to *Hin*dlll in pRTW8, was cloned into the *Smal* and *Hin*dlll digested pDML308 to yield plasmid pDML309.

Finally the *Amp*^{R} selection marker in pDML309 was replaced by the Km^R gene. This was achieved by inserting the Klenow filled-in *Mlul* fragment of pBGS18+ containing the *Km*^{R} gene in the *Amp*^{R} gene *Hin*cl site. The isolated and purified plasmid was called pDML310. This plasmid was used to transform *E. coli* JM105 and the resulting strain produced and exported in the periplasm a 262 amino acid polypeptide referred to BlaR-CTD and corresponding to the last residues of BlaR (M^{346}-R^{601}), proceeded by the hexapeptide QEGTSP as described previously by Joris *et al.* (1990).

Protein production and purification

Fifteen litres of LB medium (1% bacto-tryptone, 0.5% bacto-yeast extract, 1% NaCl) supplemented with 50 µg ml^{-1} kanamycin were inoculated with 250 ml of a 18 h preculture of *E. coli* JM105 harbouring pDML310. The culture was grown at 37°C and under agitation (500 r.p.m.). When the A^{600} value reached 1.7, 20 µM isopropyl-β-D-thiogalactopyranoside was added and the culture continued for 1 h. Cells were collected, suspended in one litre of protoplasting buffer (30 mM Tris-HCl buffer pH 8, containing 27% sucrose (w/v), 5 mM EDTA and 20 mM $CaCl_2$), and treated by lysozyme (0.1 mg ml^{-1}). Protoplasts were eliminated by centrifugation and the supernatant containing the periplasmic fraction dialysed against 30 mM Tris-HCl pH 8. The solution was loaded onto a Q Sepharose Fast flow (Amersham Pharmacia Biotech) column (2.6 × 30 cm) and BlaR-CTD was eluted with a linear (0-0.5 M) NaCI gradient over 1 litre at a flow rate of 5 ml min^{-1}. After concentration of the BlaR-CTD containing fractions, the receptor was further purified by affinity chromatography on an agarose-phenyl-boronate column (type B) (2.5 × 20 cm) (Cartwright and Waley, 1984) equilibrated in 0.1 M Trisphosphate, pH 8.6, on which BlaR-CTD was selectively retarded. During the purification steps, the presence of the expected protein was detected by SDS-polyacrylamide gel electrophoresis followed by Coomassie blue staining and the purity of the final preparation estimated by the same method and by titration with [^{14}C]-benzylpenicillin.

Kinetic analysis

Due to the very low k_3 values observed for the interaction of BlaR-CTD with β-lactam antibiotics, the methods used for the determination of the kinetic parameters are those described for the study of penicillin-binding proteins (Frère *et al,* 1992). All experiments were performed at 37°C in 50 mM sodium phosphate buffer, pH 7. In these conditions BlaR-CTD is stable for at least 20 h.

Determination of k_2/K-values

Direct determination. Due to the high k_2/K' value for the acylation of BlaR-CTD by benzylpenicillin the experiment was performed in a quenched-flow apparatus (QFM-5, Biologic). In this experiment, the β-lactam concentration was equal to that of BlaR-CTD and the analysis performed according to the general second order equation.

100 µl of 4 µM BlaR-CTD were mixed with 100 µl of 4 µM [^{14}C]benzylpenicillin (2.5 mCi mmole^{-1}, Amersham Pharmacia Biotech) and allowed to react for periods of time ranging from 70 ms to 10 s before the reaction was stopped by adding 33 µl of 6 N HCl. The acidified mixture was added with 60 µl of ice-cold 50% (w/v) trichloroacetic acid containing 3.75% (w/v) tungstosilicic acid to precipitate BlaR-CTD (Martin and Waley, 1988). After incubation at 4°C for 2 min, the protein was isolated by centrifugation, washed with water and redissolved in 0.8 ml of 1% SDS in 0.1 M sodium acetate buffer, pH 4.5. Thereafter, the quantity of acylated-BlaR-CTD was quantified by counting the radioactivity.

Competition method. The k_2/K' values for the other β-lactams were determined by direct competition with [^{14}C]-benzylpenicillin (Frère *et al.,* 1992).

BlaR-CTD (2.5 µM) was added with a mixture of [^{14}C]benzylpenicillin (Bpen) and another unlabelled antibiotic (Una) in a concentration ratio such that, at the end of a 5 min incubation, [E-Una*] ≅ [E-Bpen*] ≅ [BlaR-CTD]/2 in which, E-Una* and E-Bpen* are the adducts resulting from the acylation of BlaR-CTD by the unlabelled antibotic or [^{14}C]benzylpenicillin respectively. The protein was precipitated by addition of the TCA-tungstosilicic acid mixture and the radioactivity of the solubilised precipitate determined. The k_2/K' value for the unlabelled antibiotic is given by the following equation (Frère *et al,* 1992): $[E\text{-}Una^*]/[E\text{-}Bpen^*] = (k_2/K')_{una} [Una]_0/(k_2/K')_{Bpen}[Bpen]_0$, in which $[Una]_0$ and $[Bpen]_0$ are the initial concentrations of the unlabelled antibiotic and benzylpenicillin respectively. The value of [E-Una*] was obtained as $[E\text{-}Bpen^*]_0$ - [E-Bpen*], where $[E\text{-}Bpen^*]_0$ is the concentration of acylated-BlaR-CTD in the absence of competing unlabelled antibiotic.

Determination of k3. With both labelled and unlabelled antibiotics, the degradation of acylated-BlaR-CTD obeyed simple first-order kinetics. The k_3 value for benzylpenicillin was first determined with the labelled compound. 0.3 nmoles BlaR-CTD were reacted with 0.25 nmoles [^{14}C]benzylpenicillin in a total volume of 200 µl. The mixture was incubated at 37°C and the quantity of residual acylated-BlaR-CTD was determined after various periods of time by TCA-tungstosilicic acid precipitation as described above.

Unlabelled compounds were added with a twofold molar excess of BlaR-CTD (3 µM) and the acylated BlaR-CTD was left to decay for increasing periods of time. The regeneration of free BlaR-CTD was quantified by counter-labelling with 5 µM [^{14}C]benzylpenicillin] and precipitation as above.

Induction

Cultures of *B. licheniformis* 749/I grown in LB broth with shaking at 37°C to early log phase (A^{550} = 0.1) were induced by adding benzylpenicillin or 7-minocephalosporanic (7-ACA) and allowing growth to continue. At various times after induction, samples of the culture were gently centrifuged and the β-lactamase activity was assayed by monitoring the hydrolysis of 100 µM nitrocefin. In these conditions, one µg of β-lactamase hydrolyses 0.68 µmole of nitrocefin per min (Matagne *et al,* 1990).

To induce the cells during short times with benzylpenicillin, the excess of antibiotic was hydrolysed by addition of the *Bacillus cereus* Zn^{++}-β-lactamase (final concentration: 40 µg ml^{-1}) and 100 µM $ZnCl_2$. To quantify the induced β-lactamase, EDTA (final concentration: 33 mM) was added to inactivate the Zn^{++}-β-lactamase and the remaining β-lactamase activity determined as above.

Northern blotting analysis

Preparation of antisense RNA probe. The 0.785 kbp DNA sequence of the *blaP* gene was amplified by polymerase chain reaction using pDML995 (Filée *et al.* 2002) as template and the following oligonucleotides as primers: 5'-GCAGTGTTGCTTTTCTCTTGCGTCGCG-3' and 5'-CTGCAAGAACGACAGGATCTCCTTTTGGCG-3'. This amplified fragment was ligated to T7 phage RNA

polymerase promoter using Lig'nScribe™ Kit (Ambion). The Maxiscript™ Kit (Ambion) containing the T7 RNA polymerase was after used to obtain the RNA probe containing biotinylated UTP (Biotin RNA labeling Mix, Ambion).

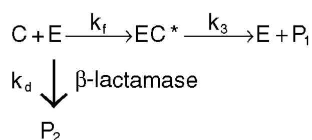
RNA isolation and purification. Total RNAs were isolated from differents samples of cultured *B. licheniformis* 749/I by using the SV Total RNA Isolation System Kit (Promega) and following instructions of the manufacturer. MicrobExpress™ Kit (Ambion) was used to enrich bacterial mRNAs from purified total RNAs by removing the 16S and 23S ribosomal RNAs.

The yield of total mRNAs obtained was determined spectrophotometrically at 260 nm with a GeneQuant RNA/DNA calculator (Amersham Biosciences), where 1 absorbance unit corresponding to 40 µg of single stranded RNA ml^{-1}.

Northern blotting. Northern blotting was realised with NorthernMax™ Kit (Ambion). 0.75 µg of mRNA were separated by an agarose gel electrophoresis. BrightStar™ Biotinylated RNA Millenium Markers™ (Ambion) was used as reference. After electrophoresis, mRNA were transferred with VacuGene XL Vacuum Blotting System (Amersham Biosciences) on a BrightStar™-Plus positively charged nylon membrane (Ambion). After 3.5 h of transfer, the membrane was hybridized with the biotinylated RNA probe during 17 h at 68°C in a HB-1D Hybridization Incubator (Techne USA). The Bright-Star™ Biodetect™ Kit (Ambion) was used to detect the biotinylated probe by using a chemiluminescent detection process including streptavidin, biotinylated alkaline phosphatase and CDP-Star.

Simulations

Simulations of residual β-lactam concentrations and of the ratios of free/acylated (i.e. active/inactive PBP1) in growing cells were performed as described by Lepage *et al.* (1995a) and on the basis of the simple model:



where E is PBP1, C the antibiotic, P_2 the β-lactamase mediated hydrolysis product and EC* the acylated PBP1.*kf* $\text{PBP1}.kf = \frac{k_2}{K}.[C]$ and *kd* is the first-order rate constant for the hydrolysis of β-lactam at a concentration much lower than the Km value and corresponds to $\frac{k_2.Bo}{K'}$ where *Bo* is the concentration of β-lactamase.

At β-lactam concentrations which did not affect the growth curve, it was assumed that the rate of production of PBP1 corresponded to that of cell multiplication, i.e. 0.46×10^{-3} s^{-1} for a generation time of 25 min. When growth was affected, this value was decreased to 0.23×10^{-3} s^{-1} or 0.46×10^{-4} s^{-1} according the severity of the observed effect. The basal unin-duced level of β-lactamase concentration in the culture (secreted + cell-bound) was 4-8 µg l^{-1} in the presence of benzylpenicillin (see Fig. 1). In the presence of 7-ACA, the level of β-lactamase was taken at that observed after full induction (1 mg l^{-1}). This latter value is clearly exaggerated, but 7-ACA is such a poor substrate that its concentration is barely affected. Because in all cases, the antibiotic concentrations were well below the β-lactamase Km values (Matagne *et al,* 1990). The disappearance of both benzylpenicillin and 7-ACA hydrolysis could be considered as pseudo-first order processes. On the basis of the kinetic parameters measured by Matagne *et al.* (1990), the k_d values were 3 and 6×10^{-3} s^{-1} (benzylpenicillin with 4 and 8 µg of β-lactamase l^{-1}, respectively) and 1×10^{-5} s^{-1} (7-ACA with 1 mg β-lactamase l^{-1}). The k_f and k_3 values for PBP1 were those found by Lepage *et al.* (1995a) to explain the rate of PBP1 acylation in growing cells of *B. licheniformis.*

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