

Title	Penicillin-binding proteins (PBP) and Lmo0441 (a PBP-like protein) play a role in beta-lactam sensitivity of <i>Listeria monocytogenes</i>
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Publication date	2009-12-15
Original Citation	Van de Velde, S. and Carryn, S. and Van Bambeke, F. and Hill, C. and Tulkens, P.M. and Sleator, R.D. (2009) 'Penicillin-binding Proteins (PBP) and Lmo0441 (a PBP-like protein) play a role in Beta-lactam sensitivity of <i>Listeria monocytogenes</i> '. <i>Gut pathogens</i> , 1:23 doi: 10.1186/1757-4749-1-23
Type of publication	Article (peer-reviewed)
Link to publisher's version	http://www.gutpathogens.com/content/1/1/23 - 10.1186/1757-4749-1-23
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Download date	2024-04-25 10:11:54
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Short report

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Penicillin-binding Proteins (PBP) and Lmo0441 (a PBP-like protein) play a role in Beta-lactam sensitivity of *Listeria monocytogenes*

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Published: 15 December 2009

Received: 3 December 2009

Gut Pathogens 2009, 1:23 doi:10.1186/1757-4749-1-23

Accepted: 15 December 2009

This article is available from: <http://www.gutpathogens.com/content/1/1/23>

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Abstract

While seven penicillin-binding proteins (PBPs) or PBP-like proteins have been identified either by radiolabelled penicillin binding studies or genomic analysis, only PBP3 has been considered of interest for Beta-lactams activity against *Listeria monocytogenes*. Herein we reveal that both PBP4 and Lmo0441 (a PBP-like protein) play a direct role in cephalosporin activity in *L. monocytogenes* while PBP4 additionally has a protective affect against both penicillin and carbapenem.

Findings

The Gram-positive foodborne pathogen *Listeria monocytogenes* is a causative agent of gastroenteritis [1] and in severe cases, listeriosis, which ranges from a mild flu-like illness to meningitis in non-pregnant individuals, or as infection of the foetus in pregnant women [2]. Ampicillin, either alone or in combination with gentamicin, remains the gold standard treatment for *L. monocytogenes* infection [3]. Conversely, cephalosporins are usually poorly active and thus not recommended in the treatment of listeriosis [4,5]. This dissociation in susceptibilities between β -lactams has been rationalized by the observation that cephalosporins have poor affinity for PBP3 compared to penicillin, suggesting that PBP3 is the primary lethal target for β -lactams in *L. monocytogenes* [6]. However, at least five different PBPs have been identified in *L. monocytogenes* based on their ability to bind radioactive penicillin [6,7]. Moreover, genome analysis revealed seven distinct genes with homologies to PBPs in *L. monocytogenes* [8]. With the

exception of *pbpB*, each of the remaining genes has been disrupted by insertional mutagenesis [9] without loss of cell viability, suggesting that they are not critically required for normal synthesis of cell wall peptidoglycan. Based on a previous study by Guinane et al., [9] in which an increase in the activity of cephalosporins was observed for *L. monocytogenes* disrupted in *lmo2229* and *lmo0441*, we investigated the role of their corresponding proteins (PBP4 and Lmo0441 respectively) in protecting the pathogen against a penicillin (ampicillin, AMP), a carbapenem (meropenem, MEM) and a cephalosporin (cefuroxime, CFX).

The growth rates for wild-type and mutant strains, together with time profiles for the activity of AMP, MEM and CFX at 100 times their MIC were tested over a 24 h period in TSB broth (Fig. 1, left panel). No significant differences in the growth rates were observed between wild type and both mutant strains. However, this effect is only

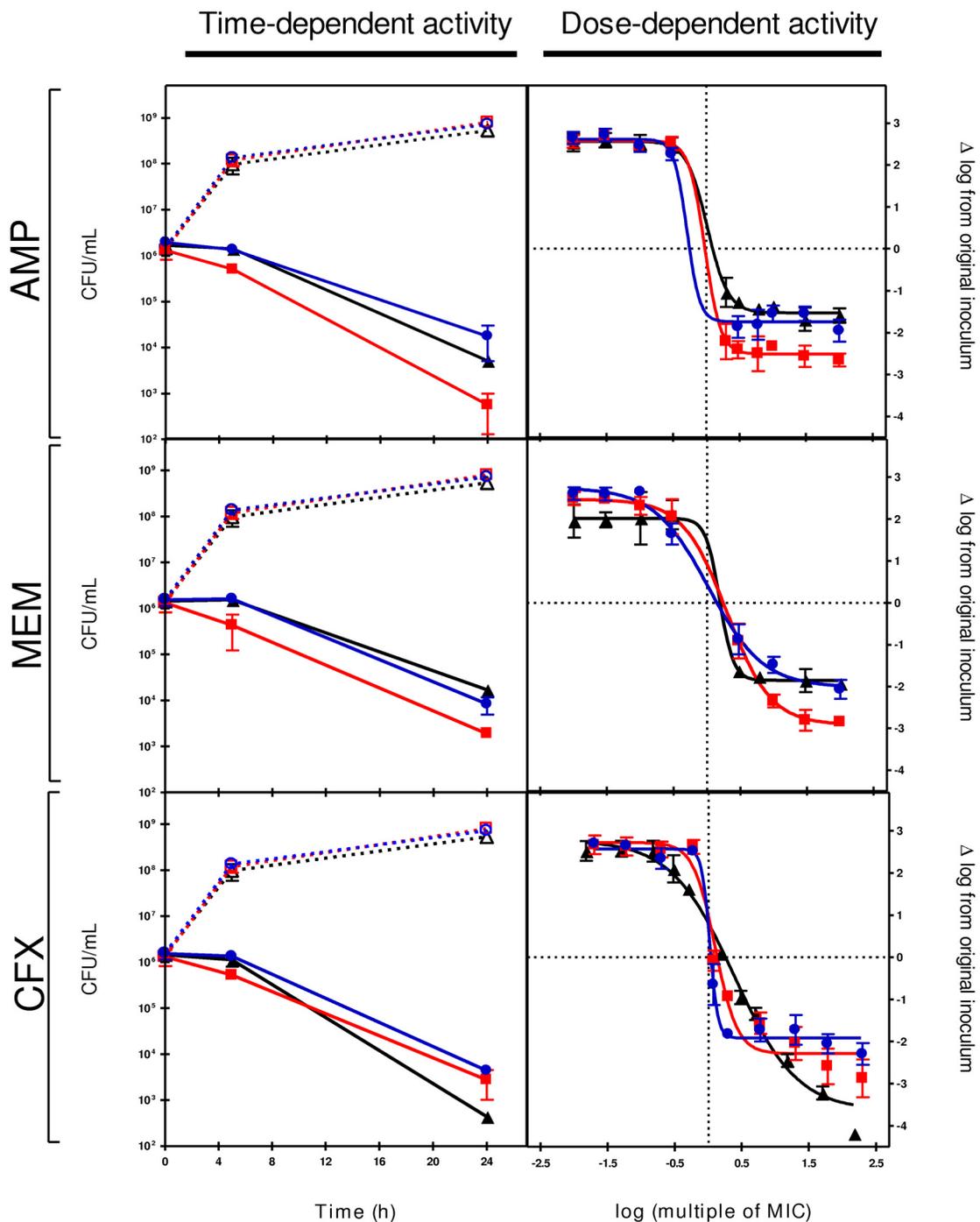


Figure 1
Activities of ampicillin (AMP), meropenem (MEM) and cefuroxime (CFX,) were tested against EGDe wild-type (triangle), ΔPBP4 (squares) and ΔLmo0441 (circle). Left panel shows bacterial growth (open symbols, discontinuous lines) and antibiotic activity (closed symbols and continuous lines) during 24h at 100 times MIC in TSB broth. Right panel shows, at 24h, the dose response curves of the 3 same antibiotics in TSB broth. Curve fitting and statistical analyses were performed using GraphPad Prism for windows version 4.03 and GraphPad Instat version 3.01 (GraphPad® Software, San Diego, CA).

Table 1: MIC measurements, regression parameters^a and statistical analysis of the dose-response curves illustrated in Figure 1

	AMP			MEM			CFX		
	MIC (mg/L)	E_{max}^b (CI) ^c $\Delta\log$ from original inoculum	R ²	MIC (mg/L)	E_{max}^b (CI) ^c $\Delta\log$ from original inoculum	R ²	MIC (mg/L)	E_{max}^b (CI) ^c \log from original inoculum	R ²
EGDe WT	0.125	-1.53 (-1.7 to -1.3)	0.97	0.0625	-1.85 (-2.2 to -1.5)	0.95	8	-3.65 (-4.2 to -3.1)	0.97
ΔPBP4	0.125	-2.51 (-2.7 to -2.3) ^d	0.98	0.0625	-2.92 (-3.3 to -2.5) ^d	0.98	2	-2.29 (-2.6 to -1.9) ^d	0.93
ΔLmo0441	0.125	-1.74 (-1.9 to -1.5)	0.97	0.0625	-2.02 (-2.5 to -1.6)	0.97	2	-1.92 (-2.2 to -1.7) ^d	0.95

Ampicillin (AMP), meropenem (MEM) and cefuroxime (CFX) MICs measurements for the parental strain EGDe and Δ PBP4 and Δ Lmo0441 mutant strains. MICs of AMP and MEM, with a respective value of 0.125 and 0.0625 mg/L, did not differ between wild type and mutant strains. However, there is a 4-fold increase in MIC of CFX for both mutant strains, which passes from 8 mg/L to 2 mg/L, a finding which is in agreement with previous observations [9].

^a Regression parameters are based on the Hill equation (using a variable slope)

^b Concentration causing maximal activities (E_{max} values), as obtained from the Hill equation. Values are expressed as a multiple of the respective MIC for a given strain.

^c CI - Confidence Intervals (95%)

^d Significantly different ($p < 0.05$) from the wild type strain as calculated by F-test

observed after an incubation of 24 h, the Δ PBP4 strain appeared significantly more sensitive than the wild type to both AMP and MEM, a phenotype not observed for the Δ Lmo0441 strain. In contrast, both PBP mutant strains appeared less sensitive to CFX, after 24h, when compared to the wild type strain, an unexpected result given their MICs.

To better understand the role of PBP4 and Lmo0441 proteins, full dose-response experiments were run with AMP, MEM and CFX using a wide range of concentrations and are presented in Fig. 1, right panel. Data collected were used to determine the Hill function regression parameters allowing the determination of the relative drug efficacy (E_{max}) presented in Table 1. An increase of drug efficacy was observed for AMP and MEM against the Δ PBP4 strain (1.64 and 1.58 times the E_{max} of the parental wild-type strain respectively); an effect which could not have been predicted from MIC measurements; while E_{max} of AMP and MEM were not affected in the Δ Lmo0441 strain. In contrast to the results obtained for AMP and MEM, both PBP mutant strains were affected by the activity of CFX and in this case, a decrease of drug efficacy was observed with the use of Δ PBP4 and Δ Lmo0441 strains (0.63 and 0.53 times the E_{max} of the wild-type strain respectively).

In conclusion then, the construction of PBP mutant strains Δ PBP4 and Δ Lmo0441 (Fig. 2) displayed no obvious growth defects in *L. monocytogenes*, suggesting that the individual PBPs are most likely not essential for growth under normal growth conditions. Furthermore, we demonstrate a role for PBP4 in penicillin and carbapenem activities in *L. monocytogenes*. Indeed, dose-response experiments over a wide concentration range reveals an increase in sensitivity of the Δ PBP4 strain for both AMP and MEM compared to the parental wild-type strain. This

observation, which could not have been inferred from MIC measurements alone, underscores the importance of accurate techniques to predict antibiotic activity. Moreover, this effect appears specific to the Δ PBP4 strain and is not apparent in the Δ Lmo0441 mutant. The observed protective effect of PBP4 against penicillin and carbapenem activities may result from decreased competition between PBPs or from the loss of cooperative action with other PBPs for the formation of the cell wall peptidoglycan.

This study also reveals that loss of PBP4 or Lmo0441 proteins allows a decrease of maximal activity of cephalosporin, a class of antibiotics which, due to their poor affinity for the PBP3, are not particularly effective against *L. monocytogenes* [6]. This observation, which runs con-

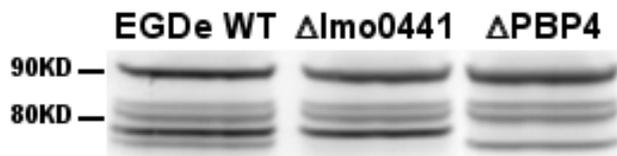


Figure 2
Western Blot analysis of PBPs was performed as described previously [10] with minor modifications. 10 ml of log phase bacteria ($OD_{660\text{ nm}} = 0.5$) were exposed to Bocillin FL (25 μg) for 30 min at 37°C. Bacteria were then harvested by centrifugation, washed four times with phosphate-buffered saline, and lysed by freeze-thawing process. Proteins were separated by 1D SDS-PAGE, and the acyl-enzyme complex was detected (excitation: 488nm, emission: 520nm) with a fluorescent scanner (Typhoon 9410, GE Healthcare). Mutagenesis results in a loss of bands of approximately 75 and 78 KDa which correspond to the molecular weight of PBP4 and Lmo0441 proteins respectively.

trary to what was observed for AMP and MEM may be explained by the fact that PBP3 may increase its relative action in peptidoglycan synthesis when PBP4 or Lmo0441 are deleted: conferring resistance to cephalosporin. Thus, even if PBP3 remains the principal target of β -lactam antibiotics, as is the current dictum, PBP4 and Lmo0441 also appear to play an important role in the activity of β -lactam antibiotics.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SVDV, PMT, CH and RDS conceived of the study and drafted the manuscript. SVDV, SC and FVB carried out the work. All authors read the manuscript and approved the final draft.

Acknowledgements

We are grateful to Charlotte Misson who provided skillful technical assistance and the manufacturers for the kind gift of their antibiotics. This work was supported by a FIRST-Doctorate fellowship awarded to SVDV by the *Direction Générale de la Recherche et des Technologies of the Région Wallonne*, Belgium and co-financed by Eumedica s.a.. RDS is a Health Research Board (HRB) and Alimentary Pharmabiotic Centre (APC) Principal Investigator.

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