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BIOCHEMICAL CHARACTERIZATION OF L1 AND L2 B-LACTAMASES FROM CLINICAL ISOLATES OF *STENOTROPHOMONAS MALTOPHILIA*

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ABSTRACT (Revised)

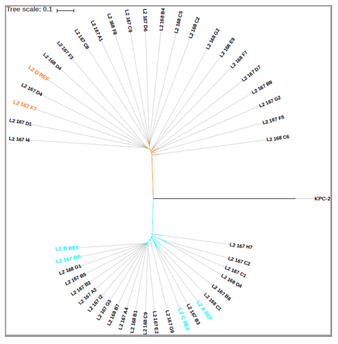
Background: *Stenotrophomonas maltophilia* is a Gram-negative, non-fermenting opportunistic pathogen. Two β-lactamases provide intrinsic resistance to β-lactams: a class B Metallo- β-lactamase L1, and a class A serine β-lactamase (SβL) L2. Recently, we described novel variants of the L1 and L2 in a collection of clinical *S. maltophilia* isolates collected in the US, and showed through analyses of the amino acid sequences that L1 and L2 grouped into 4 (A-D, B, C, and E) and 2 (A and D) clades, respectively. We aimed to characterize the new L1 and L2 clinical variants biochemically. **Methods:** Representative bla_{L1} and bla_{L2} genes from each of the identified clades were cloned into pBC-SK (+) and pET-26 vectors and transformed into *E. coli* DH10B and BL21 (DE3) cells, respectively. Minimal inhibitory concentrations (MICs) were determined using CLSI approved methods. Cell-based assays and biochemical characterization performed on purified enzymes, including circular dichroism (CD), thermal stability, and steady-state kinetics assays, were performed. **Results:** L1 variants conferred the same level of resistance to carbapenems and displayed different tolerances to Zn starvation. L2B granted higher MICs to 3rd gen cephalosporins and aztreonam than L2D. Kinetics assays confirmed differences in the k_{cat} of both enzymes to ceftazidime ($32s^{-1}$ for L2B vs. $7s^{-1}$ for L2D) and avibactam inhibition constant K_i (1.7 μM for L2B vs. 4.5 μM for L2D). Structurally, L2B and L2D present distinctive CD spectra and thermal stabilities (ΔTm 5°C).
Conclusions: Carbapenemase activity is conserved among L1 variants, and have different tolerance to Zn(II) scarcity. Differences between L2B and L2D might have arisen due to the use of cephalosporins and SβL inhibitors. Further experiments are on the way to determine the structural basis of these observations and the implication of these for the design of novel β-lactamase inhibitors.

BACKGROUND

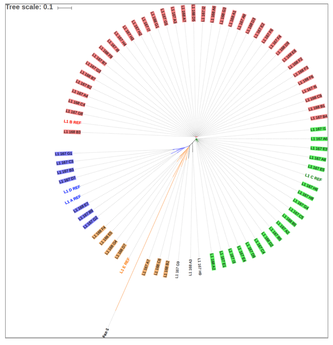
- *Stenotrophomonas maltophilia* is an aerobic, non-fermentative, trans-kingdom Gram-negative pathogen [1], classified by the WHO as a **leading nosocomial, multidrug resistant** organism [2]
- Intrinsic β-lactam resistance due to the expression of two inducible **β-lactamases:**
  - **L1** a class B3 Metallo-β-lactamase
  - **L2** a class A, clavulanate-susceptible cephalosporinase

OBSERVATIONS

Analysis of the deduced amino acid sequences obtained from 116 clinical strains identified **43 new clinical variants of L2,** that group into two different clades [3].



Analysis of the deduced amino acid sequences obtained from 73 clinical strains identified **34 new clinical variants of L1,** that group into four different clades [3].



RESEARCH QUESTIONS

Are there biochemical differences among the variants? Is this L1 and L2 sequence diversity reflected in different hydrolytic profiles?

RESEARCH DESIGN

1. bla_{L1} and bla_{L2} cloning of representative clinical enzymes:
   - into pBC SK for cell-based assays
   - into pET-26 for expression and purification
2. Agar dilution minimum inhibitory concentration (MIC) assays
3. Steady-state kinetics
4. Circular dichroism
5. Molecular modeling

RESULTS

1. Representative sequences of each clade (**L2B** and **L2D**) were cloned. Differences in the Ω-loop are indicated.

```
L2Bxxx0    RASGDTVSRSDRLEPELNSFAKGDPRDTTT
L2Dxxx1    RGQGDSITRNDRNEPDVNLFAKGDPRDTTS
                              Ω-loop
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2. **L2B** and **L2D** expressed in *E. coli* DH10B confer ESBL and inhibitor resistant phenotypes (**Table 1**).

Table 1. MICs of *E. coli* pBCSK producing L2B and L2D

	AMP	PIP	ATM	CAZ	CTX	CRO	FEP	TIM	SAM	TZP	CZA	IPM
pBCSK+	8	4	0.25	0.5	0.125	0.125	0.125	4	4	2	0.25	0.5
L2B pBCSK	4096	512	**2048**	**128**	**32**	32	2	256	64	512	0.5	0.5
L2D pBCSK	4096	512	**512**	**8**	**8**	16	2	128	128	256	0.25	0.5
R Breakpoint	≥32	≥128	≥16	≥16	≥ 4	≥ 4	≥16	≥ 128/2	≥32/16	≥128/4	≥16/4	≥4

AMP, ampicillin; PIP, piperacillin; ATM, aztreonam; CAZ, ceftazidime; CTX, cefotaxime; CRO, ceftriaxone; FEP, cefepime; TIM, ticarcillin-clavulanate; CZA, ceftazidime-avibactam; IPM, imipenem.

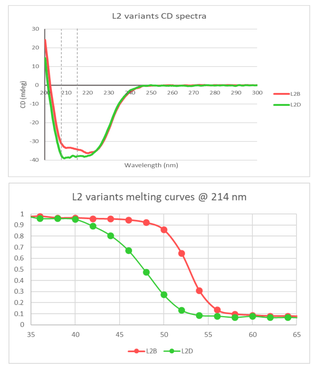
3. In line with the cell-based assays, **kinetic assays** confirmed that **L2B** displays higher catalytic efficiencies than **L2D** (**Table 2**).

Table 2. Kinetic parameters of L2B and L2D

Substrate	L2	K_M	k_{cat} (s^{-1})	k_{cat}/K_M ($\mu M^{-1}s^{-1}$)
Cefotaxime	B	9.4	0.8	2
	D	-	-	1*
Ceftazidime	B	-	-	0.06*
	D	-	-	0.01*
Nitrocefin	B	85	1055	12.4
	D	78	730	9.4
Imipenem	B	-	-	0.001*
	D	-	-	0.0004*

* Calculated from the progress curves of reaction. Values reported are averages of triplicate experiments. Errors were less than 10%

4. **Figure 1. A)** L2B and L2D CD spectra. **B)** Melting point differences indicate that L2D is less stable than L2B (46±1 °C vs. 52±1 °C, respectively).



5. As shown in **Figure 2**, an overlay of L1A (PDB 2MF6) and a model of L1E, synonymous and non-synonymous substitutions are distributed throughout the structure

Substitutions at the active site loops (shown in sticks) are predicted to affect substrate binding, reducing the affinity for certain substrates

6. Sequences of previously characterized L1 variants (**L1 A-E;** [4]) representative of each clade were cloned. **L1 variants** expressed in *E. coli* DH10B confer similar resistance towards carbapenems. L1E is weaker at conferring resistance to cephalosporins (**Table 3**).

Notably, L1E performs worse than any other variant under low Zn(II) availability (**Figure 3**).

Figure 2. Overlay of L1A (PDB 2MF6) and a model of L1E

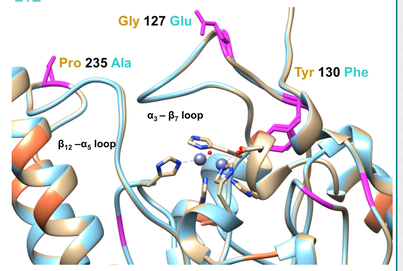


Table 3. MICs of *E. coli* pBCSK producing L1 variants

	PIP	CAZ	CTX	FEP	IPM	MEM
pBCSK+	4	0.5	0.125	0.06	0.5	≤0.125
L1a	2048	512	64	4	32	32
L1b	2048	512	64	4	16	16
L1c	2048	512	128	4	16	16
L1d	1024	512	64	4	8	16
L1e	1024	**16**	**16**	**0.5**	32	32

PIP, piperacillin; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; IPM, imipenem; MEM, meropenem.

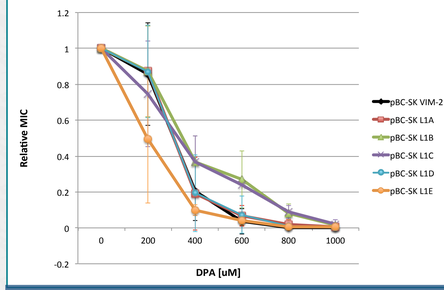


Figure 3. L1 variants display different tolerances to Zn(II) starvation. Imipenem MICs of *E. coli* DH10B cells expressing L1 variants in MH agar supplemented with the indicated concentrations of DPA relative to the MIC in 0 μM DPA. Data shown is the mean of biological replicates ± standard error. VIM-2 is shown as a reference.

CONCLUSIONS

- Representative clinical variants of L2 confer ESBL and inhibitor resistant phenotypes, except to avibactam
- Carbapenemase activity is conserved among L1 variants, and they display different tolerance to Zn(II) scarcity
- The implication of these observations for the design of novel β-lactamase inhibitors remains to be determined

ACKNOWLEDGMENTS

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