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Impact of Inducible bla_{DHA-1} on Susceptibility of *Klebsiella pneumoniae* Clinical Isolates to LYS228 and Identification of Chromosomal *mpl* and *ampD* Mutations Mediating Upregulation of Plasmid-Borne bla_{DHA-1} Expression

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ABSTRACT Twenty-three *Klebsiella pneumoniae* (bla_{DHA-1}) clinical isolates exhibited a range of susceptibilities to LYS228, with MICs of ≥8 μg/ml for 9 of these. Mutants with decreased susceptibility to LYS228 and upregulated expression of bla_{DHA-1} were selected from representative isolates. These had mutations in the chromosomal peptidoglycan recycling gene *mpl* or *ampD*. Preexisting *mpl* mutations were also found in some of the clinical isolates examined, and these had strongly upregulated expression of bla_{DHA-1}.

KEYWORDS DHA-1, *Klebsiella*, LYS228, beta-lactamases, peptidoglycan, plasmid

Resistance to β-lactams often results from the expression of ever-evolving serine β-lactamase enzymes (SBLs) that degrade penicillins, cephalosporins, monobactams, and, in some cases, carbapenems (1). Carbapenem-resistant *Enterobacteriaceae* (CRE) are increasingly regarded as a public health issue (2, 3). β-Lactamase inhibitors (BLIs) counter the effect of SBLs, but metallo-β-lactamases (MBLs) such as New Delhi metallo-β-lactamase-1 (NDM-1), usually expressed together with SBLs, have emerged in *Klebsiella pneumoniae* and *Escherichia coli* (4). No inhibitors of MBLs are currently available clinically (5). The monobactam aztreonam (ATM) is stable to MBLs (6, 7) but not all SBLs. To address this, the combination of ATM with the β-lactamase inhibitor avibactam (AVI) is currently in clinical trials (ClinicalTrials registration no. NCT01689207).

We designed the monobactam LYS228, now in phase II clinical trials (ClinicalTrials registration no. NCT03354754), that is stable to both MBLs and SBLs (8). Accordingly, the MIC_{90} of LYS228 was 1 μg/ml against 271 *Enterobacteriaceae* clinical isolates, including those expressing a variety of β-lactamases (9). The MIC_{90} of LYS228 was 1 μg/ml against the 81 *K. pneumoniae* isolates included in that panel (9). Four of the 81 were annotated as having the class C β-lactamase bla_{DHA-1}. Of these, one was found to have a truncation of the bla_{DHA-1} gene (data not shown). Of the other three, two were susceptible to LYS228 (NB29263 and NB29289, MIC of 0.125 to 0.5) (Table 1), but one was less susceptible (NB29293, mode MIC of 4 μg/ml) (Table 1). *K. pneumoniae* lacks a chromosomal *ampC* but can acquire class C genes (e.g., bla_{DHA-1}) on large plasmids. Isolates with plasmid-borne bla_{DHA-1} have been reported in several geographic locations (10–24). Plasmid-borne bla_{DHA-1} likely originated from the chromosome of *Morganella morganii* and carries with it the associated *ampR* gene, making it inducible (25, 26). Inducible bla_{DHA-1} itself may not significantly affect susceptibility to many β-lactams, but combined with other mechanisms, such as porin defects (e.g., OmpK35 and/or OmpK36 in *K. pneumoniae*), it can become more important in some isolates (27, 28). These factors can complicate susceptibility testing for β-lactams, and a lack of robust methodology for identifying these isolates may result in underestimating their prevalence (19, 29–31). To explore if the variable impact of bla_{DHA-1} on susceptibility

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TABLE 1 Antibiotic susceptibilities of *K. pneumoniae* clinical isolates harboring bla_{DHA}[a]

Strain	Annotated β-lactamase(s)[b] and relevant mutations	MIC[c] μg/ml						
		LYS228	LYS228/AVI	ATM	ATM/AVI	CAZ	FEP	MEM
NB29263	DHA-1	0.5	≤0.06	2	0.25	32	0.125	0.25
NB29289	SHV-31, DHA-1, VIM-1	0.125	≤0.06	1	0.25	>64	>16	8
NB29289-CDK0033	NB29289 *mpl* (1-bp deletion, position 45), selected on LYS228	32	0.25	>64	8	>64	>16	8
NB29289-CDK0034	NB29289 *mpl* (1-bp deletion, position 275), selected on LYS228	8	≤0.06	>64	1	>64	>16	>8
NB29293	SHV-12, CTX-M-15, DHA-1	4	0.25	>64	1	>64	>16	0.5
NB29338	SHV-OSBL(b), DHA-1; *mpl* (ΔARHVGVLPADAA$_{290–302}$)	32	0.125	>64	0.25	>64	1	2
NB29352	SHV-OSBL(b), DHA-1; *mpl* (ΔAEV$_{397–399}$, LAQKAAAAE*$_{439–448}$ to RVRLRSLPSA*)	>64	1	>64	1	>64	16	4
NB29353	SHV-OSBL(b), TEM-OSBL(b), DHA-1; *mpl* (G68D)	>64	≤0.06	>64	≤0.06	>64	16	1
NB29354	SHV-12(e), DHA-1	64	≤0.06	>64	≤0.06	>64	32	8
NB29379	SHV-OSBL, TEM-OSBL; DHA-1	1	≤0.06	8	0.125	>64	0.125	0.06
NB29380	SHV-OSBL, TEM-OSBL; DHA-1	0.25	0.125	0.5	0.25	1	0.125	0.03
NB29381	SHV-OSBL, DHA-1	2	0.125	16	0.25	64	0.125	0.06
NB29382	SHV-OSBL, DHA-1	2	0.25	8	0.125	>64	0.5	0.125
NB29383	SHV-OSBL, TEM-OSBL; DHA-1	2	≤0.06	4	0.125	32	0.25	0.06
NB29384	SHV-OSBL, TEM-OSBL; DHA-1	1	≤0.06	4	0.25	32	0.125	0.06
NB29385	SHV-OSBL, DHA-1	8	0.25	32	0.5	16	4	0.25
NB29386	SHV-OSBL, DHA-1	8	0.25	32	0.5	>64	0.25	0.06
NB29387	SHV-OSBL, DHA-1	0.5	0.125	32	0.125	32	0.125	0.06
NB29388	SHV-OSBL, TEM-OSBL; DHA-1; *mpl* (D63G)	1	≤0.06	2	0.25	64	0.125	0.125
NB29389	SHV_OSBL, TEM-OSBL; DHA-1	32	≤0.06	64	1	>64	1	0.06
NB29390	SHV-OSBL, DHA-1	8	≤0.06	64	0.5	>64	1	0.125
NB29391	SHV-OSBL, DHA-1	4	0.25	8	0.25	64	1	0.06
NB29392	SHV-OSBL, DHA-1	1	≤0.06	4	0.125	16	≤0.06	0.06
NB29393	TEM-OSBL, DHA-1; *mpl* (G to A, 14 bp upstream of ATG, K131Q, L263F, V432stop)	32	0.125	>64	1	>64	1	0.125
NB29293-CDK0021	NB29293 *mpl* (D31N), selected on LYS228	>64	0.25	>64	4	>64	>16	0.5
NB29293-CDK0022	NB29293 *mpl* (W247*), selected on LYS228	>64	0.125	>64	4	>64	16	0.25
NB29289-CDK0036	NB29289 *ampD* (W7G), selected on LYS228	16	ND	>32	ND	>32	ND	4
NB29394	DHA-1	4	0.125	16	0.5	64	0.25	0.125

[a]All strains obtained were from International Health Management Associates (IHMA) except for NB29263 (Novartis collection). AVI, avibactam; CAZ, ceftazidime; FEP, cefepime; MEM, meropenem; ND, not determined.
[b]β-Lactamase annotations are from IHMA. OSBL, original-spectrum β-lactamase. The *ampR-*bla_{DHA-1} region was confirmed here by PCR.
[c]AVI used at a fixed concentration of 4 μg/ml. Susceptibility testing was performed using a broth microdilution methodology of the CLSI (41).

extended to LYS228, we expanded our panel to 23 *K. pneumoniae* clinical isolates, all having bla_{DHA-1} (among other enzymes), and determined if this panel trended toward decreased LYS228 susceptibility. The isolates originated in the United States ($n = 1$), Europe ($n = 5$), the Middle East ($n = 3$), and the Asia-Pacific ($n = 13$). The presence of the *ampR-*bla_{DHA-1} region was confirmed for all isolates by PCR and sequencing, although strain NB29381 had a C-to-A mutation 37 bp upstream of bla_{DHA-1} and NB29390 had an A-to-T mutation 74 bp upstream of bla_{DHA-1}. A range of susceptibilities to LYS228 was observed (0.125 to >64 μg/ml) (Table 1), but the LYS228 MIC was ≥8 μg/ml for 9 of the strains. Therefore, although some isolates were susceptible to LYS228, the panel was overall significantly less susceptible than the set of *K. pneumoniae* isolates that were not selected based on the presence of bla_{DHA-1} (9). We partially characterized two strains from this panel that had different susceptibilities to LYS228. *K. pneumoniae* NB29293 susceptibility was variable, with MICs ranging from 2 to 16 μg/ml, consistent with inducibility of bla_{DHA-1} (mode MIC, 4 μg/ml) (Table 1). Addition of avibactam (4 μg/ml) improved the LYS228 MIC to 0.25 μg/ml (Table 1). An intact *ompK35* porin gene could not be amplified by PCR, and the *ompK36* porin gene encoded multiple alterations to the porin protein relative to the reference strain ATCC 43816 (32 and data not shown). These defects may be additive with the effect of the inducible DHA-1, as suggested in previous reports. A second isolate, NB29289, was consistently more susceptible to LYS228 than NB29293 (MIC of 0.125 μg/ml) (Table 1). In contrast to strain NB29293, the genes encoding OmpK35 and OmpK36 only had silent mutations compared to reference strain ATCC 43816 (32). This may explain in part

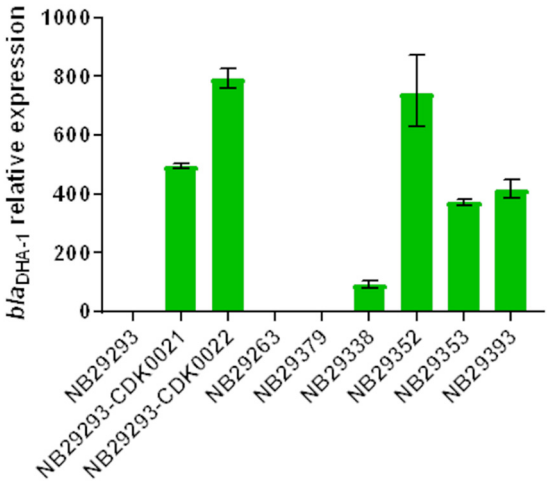

FIG 1 Constitutive bla_{DHA-1} expression in *mpl* mutants selected *in vitro* from *K. pneumoniae* NB29293 (NB29293-CDK0021 and NB29293-CDK0022) and in clinical isolates harboring alterations in *mpl* (NB29338, NB29352, NB29353, and NB29393). RT-qPCR was done as previously described (33), and expression analysis was done using the $2^{-\Delta\Delta CT}$ method (42) relative to uninduced strain NB29293. Two additional clinical isolates harboring wild-type *mpl* (NB29263 and NB29379) are included for comparison. Data are averages from two biological replicates. Induction of bla_{DHA-1} transcription (3- to 7-fold) was seen for NB29293 cells exposed to LYS228 at a MIC of 1 μg/ml (data not shown).

why NB29289 is more sensitive to LYS228 than NB29293, but this remains to be confirmed.

Consistent with DHA-1 affecting susceptibility to LYS228, mutants with constitutive upregulation of bla_{DHA-1} could be selected from strain NB29293 *in vitro* at a frequency of approximately 10^{-6} by plating cells on LB agar containing 32 μg/ml of LYS228 (8$\times$ the mode MIC). Mutant susceptibility was shifted at least 32-fold (MIC of $>$64 μg/ml). LYS228 activity was restored by the addition of 4 μg/ml avibactam, confirming involvement of a β-lactamase (NB29293-CDK0022) (Table 1). Genome sequencing of NB29293-CDK0022 using previously described methodology (33) revealed a premature stop codon (encoding W247*) in the chromosomal murein-peptide-ligase gene *mpl*, which was also present in several other mutants. An NB29293 mutant recovered from a time-kill regrowth experiment, conducted as previously described (9) (NB29293-CDK0021; Table 1), harbored a mutation in *mpl* encoding D31N. These mutants were constitutively upregulated for expression of bla_{DHA-1} (Fig. 1). Mutants were also selected from the more sensitive strain NB29289 on agar containing 1 μg/ml LYS228. Of six mutants tested, four had a 1-bp deletion (frameshift) at position 45 and one had a 1-bp deletion at position 275 of *mpl*. The MIC of LYS228 increased to 8 and 32 μg/ml for these mutants (NB29289-CDK0033 and CDK0034) (Table 1), and again LYS228 susceptibility was restored by the addition of avibactam (Table 1). It should be noted that no *mpl* mutations were isolated from several non-bla_{DHA-1} *K. pneumoniae* isolates during single-step selections described in an accompanying report (33), strengthening the association of *mpl* mutations with bla_{DHA-1}. Mpl is UDP-N-acetylmuramate:L-alanyl-γ-D-glutamyl-meso-diaminopimelate ligase, involved in peptidoglycan recycling (34, 35), and its mutational loss may induce expression of bla_{DHA-1} via changes in peptidoglycan intermediates sensed by the plasmid-encoded AmpR regulator. Upregulation of *ampC* expression caused by mutations in peptidoglycan recycling genes such as *ampD* or *ampG* in various bacteria with inducible *ampC* genes is well studied (36, 37). An *in vitro* transposon mutagenesis study also found insertions in *mpl* in *Pseudomonas aeruginosa* causing AmpR-dependent upregulation of chromosomal *ampC* and reduced susceptibility to β-lactams (38). A recent report also described the emergence of *mpl* mutations in *P. aeruginosa* during serial passaging in the presence of aztreonam (39). The remaining NB29289-derived mutant had an *ampD* encoding a previously reported W7G substitution shown to upregulate chromosomal *ampC* expression in some Gram-negative bacteria (40). The MIC of LYS228 increased to 16 for this mutant (Table 1).

The majority of the clinical isolates studied here encoded an Mpl protein identical to that of reference strain ATCC 43816 (32), which does not harbor bla_{DHA-1}. However, isolate NB29353 encoded Mpl_{G68D}, and *mpl* from isolates NB29338, NB29352, and NB29393 contained large deletions, frame shifts, and amino acid substitutions (Table 1). Expression of bla_{DHA-1} was upregulated 90-fold in NB29338 and >300-fold in NB29352, NB29353, and NB29393 relative to strains NB29293, NB29263, and NB29379, which all harbored wild-type *mpl* (Fig. 1). NB29338, NB29352, NB29353, and NB29393 were also among the least susceptible to LYS228 of the bla_{DHA-1}-containing *K. pneumoniae* strains tested (Table 1).

In conclusion, this study suggests that inducible bla_{DHA-1} can decrease susceptibility to LYS228 in some *K. pneumoniae* clinical isolates, presumably depending on the presence of additional resistance mechanisms in these strains. In cases where β-lactamases may impact the clinical utility of LYS228, pairing with an appropriate β-lactamase inhibitor may restore susceptibility. We also uncovered a novel mechanism of upregulation of plasmid-borne bla_{DHA-1} expression via chromosomal *mpl* mutations and show that these mutations occur in clinical isolates. To our knowledge, this is the first report of this mechanism in *K. pneumoniae*. Delineating the mechanism by which defects in Mpl upregulate DHA-1 expression warrants further study.

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