Trade-Offs between Antibacterial Resistance and Fitness Cost in the Production of Metallo-β-Lactamases by Enteric Bacteria Manifest as Sporadic Emergence of Carbapenem Resistance in a Clinical Setting

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ABSTRACT Meropenem is a clinically important antibacterial reserved for treatment of multiresistant infections. In meropenem-resistant bacteria of the family Enterobacterales, NDM-1 is considerably more common than IMP-1, despite both metallo-β-lactamases (MBLs) hydrolyzing meropenem with almost identical kinetics. We show that blaNDM-1 consistently confers meropenem resistance in wild-type Enterobacterales, but blaIMP-1 does not. The reason is higher blaNDM-1 expression because of its stronger promoter. However, the cost of meropenem resistance is reduced fitness of blaNDM-1-positive Enterobacterales. In parallel, from a clinical case, we identified multiple Enterobacter spp. isolates carrying a plasmid-encoded blaNDM-1 having a modified promoter region. This modification lowered MBL production to a level associated with zero fitness cost, but, consequently, the isolates were not meropenem resistant. However, we identified a Klebsiella pneumoniae isolate from this same clinical case carrying the same blaNDM-1 plasmid. This isolate was meropenem resistant despite low-level NDM-1 production because of a ramR mutation reducing envelope permeability. Overall, therefore, we show how the resistance/fitness trade-off for MBL carriage can be resolved. The result is sporadic emergence of meropenem resistance in a clinical setting.

KEYWORDS Enterobacter, Klebsiella, NDM-1, RamA, meropenem
intI, encoding an integrase enzyme, an array of gene cassettes, and a 3’ conserved sequence. Gene cassettes are promoter-less and consist of an open reading frame and an adjacent recombination site, attC, specifically recognized by the integrase enzyme. A common promoter (Pc) located within the intI sequence directs expression of all gene cassettes in an integron (9). There are essentially three strengths of Pc, PcS, strong; PcW, weak; and PcH, intermediate (10).

The blaNDM-1 gene is not a gene cassette but has been mobilized by an insertion sequence (IS) element, ISAbA125 (11). This mobilization also drives expression of blaNDM-1 because ISAbA125 carries an outward-facing promoter, Pout (12). In a recent UK study, NDM-1 was found to be the dominant MBL in carbapenem-resistant Enterobacterales clinical isolates, with IMP-1 not being found at all (13). One possible explanation is that NDM-1 is a lipoprotein and has evolved to perform well in the sort of low-zinc environment often seen at sites of infection (14), something which is enhanced in various NDM variants, particularly NDM-4 (15). However, it is possible that positive selection for NDM-1 production is driven by something more fundamental. There is some evidence that IMP-1-encoding plasmids only confer borderline resistance to carbapenems in Escherichia coli, even when zinc concentrations are high (e.g., as seen in reference 16), whereas MICs of carbapenems against E. coli transconjugants carrying NDM-1 plasmids are much higher (e.g., as seen in reference 8). We hypothesize that a more consistent ability to confer carbapenem resistance is part of the reason why NDM-1 is dominant over IMP-1 among carbapenem-resistant isolates. If correct, this would imply that the levels of active enzyme produced are frequently greater for NDM-1 than for IMP-1-positive Enterobacterales because, catalytically, the enzymes are very similar (8).

The aim of the work presented here was to test the hypothesis that NDM-1 and IMP-1 confer different carbapenem MICs because they are produced at different levels from their native expression environments and that NDM-1 more commonly confers carbapenem resistance than IMP-1. Furthermore, we have investigated the fitness trade-offs that come into play when higher-level MBL production is necessary to confer resistance. Finally, we report a clinical case demonstrating how these fitness trade-offs manifest in the real world.

RESULTS AND DISCUSSION

blaNDM-1 is expressed at higher levels than blaIMP-1 and confers meropenem resistance in Enterobacterales clinical isolates. A BLASTn search of GenBank using the nucleotide sequences of blaIMP-1 and blaNDM-1 revealed that, of entries that matched with 100% coverage and identity, E. coli ($\chi^2 = 9.82, P < 0.0005$) and Klebsiella spp. ($\chi^2 = 12.72, P < 0.0005$) are more likely to carry blaNDM-1 than blaIMP-1. This analysis is supported by global surveillance data from clinical isolates. For example, from a recent SENTRY study where, of 1,298 carbapenem-resistant Enterobacterales analyzed in 2014 to 2016, blaNDM positivity was 12.7%, while blaIMP positivity was 0.4% (17). In contrast, the non-Enterobacterales Pseudomonas spp. is more likely to carry blaIMP-1 than blaNDM-1 ($\chi^2 = 30.18, P < 0.00001$).

There may be many reasons why one gene conferring resistance to an antibacterial drug disseminates more widely than another, but we sought to test the hypothesis that blaNDM-1 is dominant over blaIMP-1 in carbapenem-resistant Enterobacterales because only blaNDM-1 reliably confers carbapenem resistance. The blaNDM-1 gene is almost exclusively found downstream of an ISAbA125 sequence, which provides an outward-facing promoter, Pout, which drives blaNDM-1 expression (11). In contrast, blaIMP-1 is encoded as an integron gene cassette (7) and so can be present downstream of several different promoter (Pc) sequences (10). Of the 26 blaIMP-1 GenBank entries involving E. coli, Klebsiella spp., and Enterobacter spp. where sufficient sequence was present to identify the Pc promoter variant, 24/26 were intermediate strength as previously defined (10), and of these, 10 were Pch1 variants (Table S1 in the supplemental material). We therefore chose to compare the impact of carrying blaIMP-1 located downstream of the Pch1 promoter with blaNDM-1.
located downstream of $P_{out}$ from ISAb125 on susceptibility to the carbapenem meropenem.

Thirteen out of 13 $bla_{NDM-1}$ Enterobacterales clinical isolate transformants tested were meropenem resistant, defined using clinical breakpoints, but only 1/13 of the $bla_{IMP-1}$ transformants was meropenem resistant (Table S2). These data support our primary hypothesis that NDM-1 more readily confers meropenem resistance than IMP-1 in the Enterobacterales.

IMP-1 and NDM-1 are, in terms of meropenem catalytic efficiency, very similar enzymes (8), so our next hypothesis was that more NDM-1 is produced than IMP-1 in cells, explaining the difference in meropenem MIC. This hypothesis was also supported experimentally; the amount of meropenem-hydrolyzing activity in cell extracts of representative $bla_{NDM-1}$ transformants of $E. coli$, Klebsiella pneumoniae, and Enterobacter (Klebsiella) aerogenes was 3- to 6-fold higher than in $bla_{IMP-1}$ transformants ($P < 0.002$ for each). As expected, elevated meropenem-hydrolyzing activity was due to greater production of NDM-1 than IMP-1 protein as measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS) proteomics (Fig. 1).

Changing the ribosome binding sequence upstream of $bla_{NDM-1}$ to be identical to that found upstream of $bla_{IMP-1}$ did not significantly reduce NDM-1 production or

![FIG 1 MBL Production in Enterobacterales carrying $bla_{IMP-1}$ or $bla_{NDM-1}$ with variant upstream sequences. MBL production was measured in K. pneumoniae, E. coli, or K. aerogenes (Enterobacter aerogenes) recombinants carrying the pSU18 cloning vector, into which had been ligated $bla_{IMP-1}$ with its upstream PhC1 promoter (dark blue bars), $bla_{NDM-1}$ with its wild-type ISAb125 promoter (red bars), $bla_{NDM-1}$ with site directed mutation to convert its ribosome binding site to be identical to that upstream of $bla_{IMP-1}$ (N RBS; light blue bars), and $bla_{NDM-1}$ synthesized to have the same upstream sequence as $bla_{NDM-1}$ (N*; purple bars). (A) Meropenem-hydrolyzing activity (nmol min$^{-1}$ mg total protein$^{-1}$) was measured in whole-cell extracts. (B) IMP-1 or NDM-1 protein abundance derived from LC-MS/MS analysis of whole-cell extracts is reported normalized to the average abundance of 30S and 50S ribosomal proteins in each extract. Data are means ± standard error of the mean; n = 3.](https://aac.asm.org/content/65/8/e02412-20.full)
meropenem-hydrolyzing activity. However, generating the N* variant by replacing the entire blaNDM-1 upstream sequence with that upstream of blaIMP-1 reduced NDM-1 production to be very similar to that of IMP-1 in all three species (Fig. 1).

The correlation between high gene expression and fitness cost when carrying blaNDM-1 is associated with amino acid starvation. We next investigated whether the greater production of NDM-1 relative to IMP-1 imposes a fitness cost. Using pairwise competition experiments where transformants directly competed over 4 days in the absence of β-lactams, we showed that there is no cost of carrying blaIMP-1 in E. coli and K. pneumoniae, but there was a significant cost of carrying blaNDM-1 in both species (Table 1).

Higher production of NDM-1 versus IMP-1 could impose a fitness cost because of depletion of resources required to make the additional MBL (e.g., amino acids, energy, and zinc), or it could be due to some toxicity that the MBL imposes, as has been seen in some cases, e.g., SPM and VIM, previously (18). To differentiate between these possibilities, we investigated the physiological impact of carrying blaIMP-1 or blaNDM-1 in E. coli. To do this, we used LC-MS/MS proteomics to quantify steady-state protein abundance differences in transformants.

Of 1,390 proteins identified and quantified in the blaIMP-1 versus plasmid-only control comparison, 66 were significantly up- or downregulated (Table S3), but chi-square analysis did not reveal clustering of these proteins into any KEGG functional group, suggesting that there is little concerted physiological response to carrying blaIMP-1 (Table S4). The blaNDM-1 versus control comparison identified and quantified 1,670 proteins, of which 88 were differentially regulated (Table S5). In this case, chi-square analysis did identify clustering (Table S6) of these regulated proteins into a specific KEGG pathway, eco00260, glycine, serine, and threonine metabolism. Upregulated proteins include the committed enzymes GlyA (19), SerA (20), ThrC (21), and IlvA, which directs these amino acids into other amino acid biosynthetic pathways (22). Therefore, production of NDM-1, which is approximately 6-fold more than production of IMP-1 in E. coli (Fig. 1), comes with a significant fitness cost (Table 1), which is associated with regulatory signals of amino acid starvation (Tables S3 to S6).

Increasing IMP-1 production increases fitness cost. To further test the hypothesis that the amount of MBL protein production is a major part of the fitness cost imposed by carrying MBL genes and to exclude any NDM-1-specific effects, we aimed to increase IMP-1 production. To do this, we turned to our recently reported blaIMP-1-synonymous lysine codon variant, IMP-1-KV, where 17 AAA lysine codons were converted to the alternative synonymous codon, AAG (23). LC-MS/MS proteomics showed that the amount of IMP-1 produced from the variant blaIMP-1-KV was 2.2-fold more (P=0.005) than from wild-type blaIMP-1 in E. coli (Fig. 2). As hypothesized, this increase in IMP-1 protein production was associated with an increase in fitness cost, which was approximately 7% per day in E. coli and approximately 20% per day in K. pneumoniae (P < 0.001 for both comparisons) (Table 1). We attempted to repeat this experiment by cloning blaIMP-1 downstream of a strong integron promoter, which drives high-level gene expression, but very few E. coli transformants were recovered. In all cases, the transformants had mutations upstream of blaIMP-1 expected to reduce gene expression, e.g., those affecting the −35 or −10 promoter sequences or the spacing in between.

| Strain          | Competition       | Mean fitness ± SEM (R) |
|-----------------|-------------------|------------------------|
| E. coli MG1655  | pSU18 vs pSUH IMP  | +4.5 ± 0.5             |
|                 | pSU18 vs pSU NDM   | −8.0 ± 0.4             |
|                 | pSU18 vs pSUH IMP-KV | −1.9 ± 0.5           |
| K. pneumoniae ECL8 | pSU18 vs pSUH IMP  | +5.9 ± 0.6             |
|                 | pSU18 vs pSU NDM   | −29.3 ± 0.7            |
|                 | pSU18 vs pSUH IMP-KV | −13.6 ± 2.2           |

Cheung et al. Antimicrobial Agents and Chemotherapy August 2021 Volume 65 Issue 8 e02412-20 aac.asm.org
Accordingly, we conclude that the fitness cost of carrying this highly expressed form of bla\textsubscript{IMP-1} is too great for transformants to bear.

**Reduced NDM-1 production due to rearrangements in the bla\textsubscript{NDM-1} promoter region explains the lack of meropenem resistance in Enterobacter spp. isolates from a clinical case.** A patient was admitted directly to the intensive care unit after developing a small bowel obstruction and an aspiration pneumonia. Bronchoalveolar lavage grew *Citrobacter freundii*, *K. pneumoniae*, and *Bacteroides vulgatus*. The patient was initially treated with piperacillin-tazobactam and azithromycin and was noted to have a strangulated inguinal hernia which was repaired. Two days after admission, the patient was escalated to meropenem due to continued fever. Vancomycin was added for a possible coagulase-negative *Staphylococcus* spp. line infection. They continued to require ventilation, and a tracheostomy was performed on day 7. By 20 days after admission, symptoms had resolved, C-reactive protein had fallen to 10 from 368 mg/liter on admission, and meropenem was stopped.

Five days later, fever restarted, and a sputum sample grew *K. pneumoniae* resistant to piperacillin-tazobactam and ciprofloxacin but extended-spectrum β-lactamase (ESBL) negative and susceptible to third-generation cephalosporins. Ceftazidime and vancomycin were started. After 6 days of ceftazidime, a routine multiresistant coliform screen of the patient’s tracheostomy site noted a ceftazidime-resistant *Enterobacter* spp. (Ent1). This was ESBL positive and had a multidrug resistance phenotype (Table S7). Due to an apparently raised meropenem MIC, a Cepheid Xpert Carba R PCR test was performed, suggesting the presence of bla\textsubscript{NDM}. Despite this, Ent1 was not meropenem resistant, and so, ceftazidime treatment was switched to meropenem. After 10 days of meropenem, the patient improved, and antibiotic therapy was discontinued. Routine screens continued to isolate *Enterobacter* spp. with the same resistance pattern and being bla\textsubscript{NDM} positive (e.g., Ent2), but 12 days after the isolation of Ent1, another routine screen identified an ESBL-negative *K. pneumoniae* which was fully resistant to meropenem (KP3), as well as to third-generation cephalosporins, piperacillin-tazobactam, and ciprofloxacin (Table S7). The Cepheid Xpert Carba also identified bla\textsubscript{NDM} in KP3. The patient, however, remained well and continued off antibiotics and was discharged to the surgical ward. Subsequent routine screens continued to identify this meropenem-resistant *K. pneumoniae* and the bla\textsubscript{NDM} positive *Enterobacter* spp. that was not meropenem resistant, and specialist infection control precautions were continued.

Whole-genome sequence (WGS) analysis of the *Enterobacter* spp. isolates Ent1 and Ent2 showed them to be *Enterobacter hormaecheii* and confirmed that bla\textsubscript{NDM-1} is present on the same IncFIB(K) plasmid in both. The plasmid was assembled into a single contig of 84,659 nucleotides (nt) carrying genes conferring resistance to amikacin/ciprofloxacin (*aacA4-cr*), rifampin (*arr-3*), co-trimoxazole (*sul1*), and streptomycin (*aadA1*), all part of the same complex class 1 integron alongside bla\textsubscript{NDM-1}. Otherwise, on the chromosome, other relevant resistance genes carried by Ent1 and Ent2 were resistant to ampicillin (bla\textsubscript{TEM-1}) and...
the expected ESBL (blaCTX-M-15). The isolates also carried chromosomal mutations in gyrA (Ser83Ile) and parC (Ser80Ile) causing ciprofloxacin resistance. Collectively, this acquired resistance genotype explains the antibiograms of Ent1 and Ent2, except for the fact that meropenem resistance should have been provided by the blaNDM-1 gene but was not.

LC-MS/MS proteomics revealed that NDM-1 production was the same in Ent1 and Ent2. The amount normalized to ribosomal proteins was 0.41 ± 0.03 (mean ± SD), which was not significantly different (P = 0.13) from the amount of IMP-1 produced from its native Pch1 promoter in blaIMP-1 transformants of E. coli and K. pneumoniae described above (0.49 ± 0.18; Fig. 1). In contrast, NDM-1 production in Ent1 and Ent2 was significantly different from (P < 0.0005) and approximately 6-fold less than NDM-1 production in transformants of E. coli and K. pneumoniae where blaNDM-1 was expressed from the typical ISAba125 Pout promoter (3.24 ± 0.69; Fig. 1). This low-level production of NDM-1 in Ent1 and Ent2 likely explains why these isolates are not meropenem resistant (MIC < 4 mg/liter), as seen for blaIMP-1 transformants (Table S2).

To explain the reason for low-level NDM-1 production in Ent1 and Ent2, we compared the sequence upstream of blaNDM-1 in these two isolates with those from E. coli IR10, the source of the recombinant plasmids used above, and from K. pneumoniae KP05-506, which is the original isolate from which blaNDM-1 was identified (8). We found a significant rearrangement immediately adjacent to the ISAba125 Pout promoter in Ent1 and Ent2 (Fig. 3). There has been an insertion of an element containing a truncated blaOXA-10 gene.

The upstream variation seen in Ent1 is rare but not unique. It matched to 14 NCBI database entries reporting isolates collected in China, Taiwan, Japan, Pakistan, and the United Kingdom (Table S8). Notably, but not commented on by the authors, an E. coli transconjugant carrying plasmid pLK78, encoding blaNDM-1 with this blaOXA-10 upstream insertion, was not meropenem resistant (24). Moreover, isolates from Pakistan where the blaOXA-10 insertion upstream of blaNDM-1 was identified in several related plasmids (25) were originally collected in 2010, and the authors noted that 53% of NDM-1-producing isolates were meropenem susceptible (26).

**Low-level NDM-1 production confers meropenem resistance in a background with reduced envelope permeability.** Isolate KP3, from the same clinical case, was meropenem resistant. LC-MS/MS proteomics analysis confirmed that KP3 produced NDM-1 at the same level as Ent1 and Ent2. WGS showed that as well as carrying blaNDM-1, aacA4-cr, sul1, arr-3, and adaA1 on an IncFIB(K) plasmid identical to that found in Ent1 and Ent2, KP3 carried blaTEM-1 and blaOXA-9 found together on a second plasmid, plus the chromosomal blaSHV-1. KP3 also has Ser83Phe and Asp87Ala mutations in GyrA plus a Ser80lle mutation in ParC explaining ciprofloxacin resistance.

The β-lactamases produced by KP3, in addition to NDM-1, cannot explain the very much higher MIC of meropenem against KP3 versus Ent1 and Ent2. Analysis of KP3 WGS data for known factors that contribute to carbapenem resistance revealed only one, that KP3 is a ramR mutant, having an 8-nt insertion into ramR after nt 126, causing a frameshift. We have shown that loss of RamR in K. pneumoniae leads to enhanced AcrAB-ToIC efflux pump production, reduced OmpK35 porin production, and enhanced carbapenem MICs in the presence of weak carbapenemases (27). Hence, this mutation in

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**FIG 3** Altered upstream sequence in Ent1 and Ent2 and KP3 versus blaNDM-1 source sequences. The Clustal Omega alignment used WGS data from two isolates carrying wild-type blaNDM-1, E. coli IR10 and K. pneumoniae KP05-506 plus the sequence shared by clinical isolates Ent1, Ent 2, and KP3. Identities across all three sequences are annotated with stars.
KP3 enhances the meropenem MIC against KP3, making it resistant despite low-level production of NDM-1 due to modification of the IS\textit{Aba}125 outward-facing promoter region by insertion of a truncated \textit{bla}\textsubscript{OXA-10}.

**Conclusions.** Overall, we have observed that modest expression of \textit{bla}\textsubscript{IMP-1} from a native intermediate-strength integron common promoter (P\textsubscript{Ch1}), which is regularly seen in \textit{bla}\textsubscript{IMP-1} clinical isolates, does not provide meropenem resistance in representative \textit{Enterobacterales} strains, but neither does it cause a fitness cost. In contrast, \textit{bla}\textsubscript{NDM-1} is expressed at higher levels from its native IS\textit{Aba}125 outward-facing promoter, and this gives higher meropenem MICs and confers resistance as defined by clinical breakpoints, but this comes with a significant fitness cost. A fitness cost associated with carrying \textit{bla}\textsubscript{NDM-1} was also found in a previous report (28). We conclude that the likely reason for this fitness cost is that NDM-1 is produced at high levels when \textit{bla}\textsubscript{NDM-1} is expressed from its native promoter. The obvious explanation is that producing a large amount of a non-native protein results in amino acid depletion, which drives the cell to switch on amino acid biosynthetic pathways, which was observed in our proteomic analysis. While this maintains the supply of amino acids for protein synthesis, it diverts carbon that would otherwise be available to other processes required for cell growth. This effect may be exaggerated in the case of NDM-1 since it is targeted to the outer membrane, where it can be lost within microvesicles (29). There was no evidence of zinc starvation stress in our proteomics data, though presumably at lower zinc concentrations, the fact that NDM-1 is a zinc-containing enzyme could exacerbate the fitness cost. Our fitness assays were performed using a medium containing 6.2 \textmu M zinc, and the broth used to perform MIC testing and proteomics contains ~4 \textmu M zinc (30). The normal human serum concentration of zinc is ~12 \textmu M (31), but clearly, long-term selection pressure on \textit{Enterobacterales} is perhaps more likely to occur outside the human body, where zinc concentrations may be very much lower, even than in our assays.

Our findings provide a real-world example of fitness/resistance trade-offs. It may be that the reason for \textit{bla}\textsubscript{NDM-1} being so common in carbapenem-resistant \textit{Enterobacterales} is repeated selective pressure via carbapenem use, driving its presence despite the cost. Alternatively, natural plasmids or certain strains carrying them, or even variant \textit{bla}\textsubscript{NDM-1} genes encoded on these plasmids, might have accumulated mutations that compensate for reduced fitness. This could come without the expense of reduced carbapenem MICs, e.g., if an NDM produced at lower levels was a variant more efficient at catalyzing the hydrolysis of meropenem. But in the case reported here, we have identified the insertion of a truncated \textit{bla}\textsubscript{OXA-10} damaging the \textit{bla}\textsubscript{NDM-1} promoter region and reducing NDM-1 production in \textit{Enterobacter} spp. isolates from a clinical case, a genetic arrangement found in commensal carriage \textit{Enterobacterales} isolates from as far back as 2010 (26).

Low-level NDM-1 producers avoid the fitness cost associated with wild-type \textit{bla}\textsubscript{NDM-1} carriage but, consequently, are not meropenem resistant, though they remain cephalosporin resistant and so are likely to be maintained in an environment where cephalosporins are used. This highlights a potential infection control issue where phenotypic meropenem resistance is necessary for a positive screening outcome. As seen here, the isolates Ent1 and Ent2 were still identified as being of interest due to extra vigilance in respect of a seriously ill patient. With less vigilance, it may have been that the only notice of the presence of an NDM-1-producing isolate in or around this patient would have been following mobilization of the \textit{bla}\textsubscript{NDM-1}-encoding plasmid into the \textit{ramR} mutant \textit{K. pneumoniae} with reduced envelope permeability to create meropenem-resistant isolate KP3. This ability of reduced envelope permeability to enhance meropenem MIC against a low-level MBL producer may also explain our finding that \textit{bla}\textsubscript{IMP-1} is more common in \textit{P. aeruginosa}, a species renowned for having much lower envelope permeability than wild-type \textit{Enterobacterales} (32). In the context of “under the radar” NDM-1 production defined here, which also relies on reduced envelope permeability, we show that sudden emergence of clinically relevant meropenem resistance can occur in a manner that is not dependent on new importation events and so cannot be prevented by standard infection control measures.
Bacteria used and susceptibility testing assays. Bacterial strains used in the study were E. coli MG1655 (33) and a collection of human clinical isolate from urine (a gift from Mandy Wooton, Public Health Laboratory for Wales), a human clinical isolate of K. aerogenes, NDM-1–producing isolates of E. coli R10 and K. pneumoniae strains SM, ECL8, and NCTC 5055 (34). Antibiotic susceptibility was determined using disc testing or broth microdilution MIC assays according to EUCAST guidelines. Cation-adjusted Mueller-Hinton broth (CAMHB) was purchased from Sigma.

Molecular biology. Creation of pSUHIMP, being the cloned bla


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\textit{IMP-1}, gene downstream of a native Pch1, was via PCR using template DNA from \textit{P. aeruginosa} clinical isolate 206-3105A (a gift from Mark Toleman, Department of Medical Microbiology, Cardiff University). For PCR, we used a forward primer targeting the 5′ end of the Pch1 promoter (5′-ACCCAGTGACATAAGCCTGTCGGTTCGAAACT-3′) and a reverse primer targeting the 5′ end of a bla


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\textit{NDM-1} gene cassette, which is downstream of \textit{bla


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\textit{IMP-1}} in this isolate (5′-ACGGGAAGTTGATAGTGATTTTG-3′). The PCR amplicon was TA cloned into the pCR2.1TOPO cloning vector (Invitrogen), removed with EcoRI, and ligated into EcoRI-linearized broad host range p15A-derived vector pSU18 (35). Site-directed mutagenesis to create pSUHIMP-KV containing 14 AAA-AAG transitions was performed using the primers and methods previously reported (23). Creation of pSUNDM, being the cloned \textit{bla


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\textit{NDM-1}}, gene downstream of its native IS\textit{aba}125 promoter in plasmid pSU18, has been reported previously (36). Site-directed mutagenesis using pSUNDM as the template was performed using the QuikChange Lightning site-directed mutagenesis kit (Agilent, UK) according to the manufacturer’s instructions. The aim was to convert the native ribosome binding site upstream of \textit{bla


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\textit{NDM-1}} (AAAAAGAAAATGGACAGATTCATCT) to be the same as that upstream of \textit{bla


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\textit{IMP-1}} (AAAAAGAAAATGGACAGATTCATCT; differences underlined), using the mutagenic primer 5′-GGGTTTTTTATGGATGAAATGAAGGAATGTAGAATCCCAGAT-3′. The resultant plasmid was named pSUNDM-N9. Switching the entire upstream sequence from the ATG of \textit{bla


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\textit{NDM-1}} to be the same as \textit{bla


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\textit{IMP-1}} was performed by gene synthesis recreating the entire pSUNDM insert sequence but with the same upstream sequence carried in pSUHIMP. The resultant plasmid was named pSUNDM-N9*

Proteomic analysis. A volume of 1 ml of overnight liquid culture was transferred to 50 ml of fresh CAMHB and incubated at 37°C until an optical density at 600 nm (\textit{OD}_{600}) of 0.5 to 0.6 was achieved. Samples were centrifuged at 4,000 rpm for 10 min at 4°C and the supernatants discarded. Cells were resuspended into lysis buffer (35 ml of 30 mM Tris-HCl, pH 8) and broken by sonication using a cycle of 1 s on, 1 s off for 3 min at amplitude of 63% using a Sonic Viba-Cel UC-50STM (Sorin & Materials Inc., Newton, CT, USA). This was followed by centrifugation at 6,000 rpm (7,000 g; Sorvall RC5B Plus using an SS-34 rotor) for 15 min at 4°C to pellet nonlysed cells. Soluble proteins were concentrated to a volume of 1 ml using centrifugal filter units (Amicon Ultra-15, 3 kDa cutoff). Then, the concentration of the proteins in each sample was measured using Bio-Rad protein assay dye reagent concentrate according to the manufacturer’s instructions and normalized. LC-MS/MS was performed and analyzed as described previously (37) using 5 μl of protein for each run. Analysis was performed in triplicate, each from a separate batch of cells. Protein abundance was normalized using the average abundance of ribosomal proteins unless stated in the text.

Measurement of meropenem hydrolysis. Twenty microliters of concentrated total cell protein (prepared and assayed for concentration as above) were transferred to 180 μl of 50 mM HEPES (pH 7.5) containing 50 μM ZnSO4 and 100 μM meropenem. Change of absorbance was monitored at 299 nm over 10 min. Specific enzyme activity (pmol meropenem hydrolyzed per milligram of protein per second) in each extract was calculated using 9,600 M/s as the extinction coefficient of meropenem and dividing enzyme activity with the total amount of protein in each assay.

Pairwise fitness cost experiments. Pairwise competition experiments were performed by using M9 minimal medium to evaluate the fitness cost of carrying pSUHIMP, pSUHIMP-KV, or pSUNDM, each relative to the carriage of the pSU18 cloning vector alone. Initially, liquid cultures of both transformants in the pairwise competition were established separately in LB broth at 37°C with shaking at 160 rpm. Then, 5 μl of each overnight liquid culture was inoculated into 10 ml M9 minimal medium separately in flasks and incubated as above for 24 h as before. After this incubation, 5 μl of each overnight M9 minimal medium was again inoculated separately into 10 ml M9 minimal medium as before and grown overnight. The next day, for each competing bacterium, 75 μl of the previous day’s culture was inoculated into fresh 15 ml M9 minimal medium to obtain a mixed culture (day 1). After 24 h of incubation, 150 μl of the mixed culture was transferred into a fresh 15 ml M9 minimal medium to obtain the day 2 culture. Then, this step was performed successively until the day 4 mixed-liquid culture was attained. For each pairwise competition experiment, the above process was carried out six times in parallel, and on each day, the CFU per milliliter of the two bacteria were counted in triplicate using LB agar selective for the cloning vector (the total count of both competitors, as both are chloramphenicol resistant) and agar containing 20 mg/liter ceftazidime (to count bacteria producing IMP-1 or NDM-1). The pSU18 containing transformant count was calculated by subtracting the pSUHIMP or pSUNDM containing transformant count from the total count of bacteria in the competition.

The fitness cost of the resistant strain relative to the sensitive strain was estimated by calculating the Malthusian parameter of the strain (M) as described (38) as \( M = \frac{1}{N_1} / N_2 \), where \( N_1 \) indicates the density of the strain at the start of the day (CFU/ml), and \( N_2 \) represents the density of the strain at the end of the day (CFU/ml). Then the selection rate for a pairwise competition is calculated as \( W = M_1/M_2 \), where \( M_1 \) represents growth of the sensitive strain, and \( M_2 \) refers to growth of the resistant strain. If \( R \) (1−W, represented as a percentage) is negative, then \( M_1 > M_2 \), which implies that the sensitive strain grows faster than the resistant strain and, as a result, has a fitness advantage and vice versa.
For each day of competition, 36 values are achieved, as for each pairwise competition, there are 6 R values, and there are 6 competitions each day (6 mixed cultures a day).

Differences in the two sets of data for each pairwise comparison were assessed using mean and standard deviation of R, and an unpaired t test (with Welch’s correction) was used to assess the statistical significance of the differences observed.

Analysis to identify clustering of differentially regulated proteins. The KEGG Mapper tool (http://www.genome.jp/kegg/tool/map_pathway2.html) was used. We searched against E. coli MG1655 (organism eco) and entered a list of the UniProt accession numbers for the differentially regulated proteins. As a control, an equal number of E. coli MG1655 UniProt accession numbers were randomly selected and entered in the KEGG Mapper as above. To determine the total number of proteins in the E. coli MG1655 proteome that fall into each KEGG, the entire UniProt MG1655 accession number list was used to seed the KEGG Mapper tool. These values were used to perform a \( \chi^2 \) analysis considering the significance of clustering of differentially regulated proteins by reference to random proteins into a KEGG functional group. To maximize specificity, the comparison with random proteins was performed 10 times, each with a different list of random proteins, and the result reported was the lowest \( \chi^2 \) value obtained across all 10 comparisons.

WGS and data analysis. Genomes were sequenced by MicrobesNG (Birmingham, UK) on a HiSeq 2500 instrument (Illumina, San Diego, CA, USA). Reads were trimmed using Trimmomatic (39) and assembled into contigs using SPAdes (40) v3.13.0 (http://cab.spbu.ru/software/spades/), and contigs were annotated using Prokka (41). The presence of plasmids and resistance genes was determined using PlasmidFinder (42) and ResFinder 2.1 (43).

Ethics statement. This project is not part of a trial or wider clinical study requiring ethical review. The patient signed to give informed consent that details of their case be referred to in a publication and for educational purposes.

Data availability. The sequence of plasmid pYUI-1, the bla\(_{IMP}\)-encoding plasmid from \( P. \) aeruginosa clinical isolate 206-3105A, has been deposited under GenBank accession number MH594579.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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We declare no conflicts of interest.

Conceived the study, M.B.A. and F.H. Collection of data, C.H.P.C., M. Alorabi, Y.T., O.M, K.J.H, and F.H., supervised by M. Albur, A.P.M., and M.B.A. Cleaning and analysis of data, C.H.P.C., M. Alorabi, Y.T., O.M, K.J.H, F.H., O.M.W., and P.B.W. supervised by M. Albur, A.P.M., and M.B.A. Initial drafting of manuscript, M. Alorabi, F.H., and M.B.A. Corrected and approved manuscript, all authors.

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