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Research Paper

Reversion of High-level Mecillinam Resistance to Susceptibility in *Escherichia coli* During Growth in Urine

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ABSTRACT

Mecillinam (amdinocillin) is a β-lactam antibiotic used to treat uncomplicated urinary tract infections (UTIs). We have previously shown that inactivation of the *Escherichia coli cysB* gene is the major cause of mecillinam resistance (Mec^R) in clinical isolates. In this study, we used different *E. coli* strains (laboratory and clinical isolates) that were Mec^R due to *cysB* mutations to determine how mecillinam susceptibility was affected during growth in urine compared to growth in the commonly used growth medium Mueller Hinton (MHB). We also examined mecillinam susceptibility when bacteria were grown in urine obtained from 48 different healthy volunteers. Metabolome analysis was done on the urine samples and the association between the mecillinam susceptibility patterns of the bacteria and urine metabolite levels was studied.

Two major findings with clinical significance are reported. First, Mec^R *E. coli cysB* mutant strains (both laboratory and clinical isolates) were always more susceptible to mecillinam when grown in urine as compared to laboratory medium, with many strains showing complete phenotypic susceptibility in urine. Second, the degree of reversion to susceptibility varied between urine samples obtained from different individuals. This difference was correlated with osmolality such that in urine with low osmolality the Mec^R mutants were more susceptible to mecillinam than in urine with high osmolality.

This is the first example describing conditional resistance where a genetically stable antibiotic resistance can be phenotypically reverted to susceptibility by metabolites present in urine. These findings have several important clinical implications regarding the use of mecillinam to treat UTIs. First, they suggest that mecillinam can be used to treat also those clinical strains that are identified as Mec^R in standard laboratory tests. Second, the results suggest that testing of mecillinam susceptibility in the laboratory ought to be performed in media that mimics urine to obtain clinically relevant susceptibility testing results. Third, these findings imply that changes in patient behavior, such as increased water intake or use of diuretics to reduce urine osmolality and increased intake of cysteine, might induce antibiotic susceptibility in an infecting Mec^R *E. coli* strain and thereby increase treatment efficiency.

1. Introduction

Antimicrobial Susceptibility Testing (AST) is necessary in order to decide on suitable treatment options for bacterial infections. Importantly, MIC tests are used to set clinical breakpoints for specific combinations of antibiotic and pathogen, which are then used by prescribers to choose a particular antibacterial regimen (Turnidge and Paterson, 2007). At the moment, healthcare industry relies heavily on the in vitro Disc diffusion and MIC testing on MHB, to perform ASTs, mainly because they are simple and high-throughput methods of estimating of antibacterial effect (Balouiri et al., 2016). But when using in vitro tests as a basis for antibiotic treatment, it is assumed that a bacterial strain that is determined to be susceptible to a specific antibiotic under laboratory conditions remains susceptible during growth within a patient, and conversely, that a strain that is resistant under laboratory conditions remains resistant (and by inference untreatable with that particular antibiotic) during an infection. However, this assumption has rarely been demonstrated experimentally and the results of recent studies are shedding more light on this by showing that several pathogens alter their susceptibility when grown under more in vivo like conditions, and thereby challenging the use of one standard susceptibility test medium.

For example, a recent study by Kubicek-Sutherland et al. suggest that phenotypic resistance can be induced by environmental conditions present in human cells and tissues. The study showed for *Salmonella enterica* and *Yersinia pseudotuberculosis* that antibiotic susceptible strains might become transiently resistant to antibiotics during growth

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in a host because the growth environment (low phosphate and Mg^{2+} in macrophages) induces a bacterial response that confers increased resistance to certain antibiotic classes, for example colistin (Kubicek-Sutherland et al., 2015). Similarly, it has been demonstrated that nitrogen oxide produced by host cells can induce bacterial resistance to aminoglycosides by blocking respiration and the energy-dependent phases of aminoglycoside uptake, thereby reducing drug susceptibility (McCollister et al., 2011). This finding implies that host inflammatory responses associated with infection can promote bacterial resistance to aminoglycosides.

Two recently published studies from Ersoy et al. and Lin et al. problematize the fact that the healthcare industry relies on the single in vitro bioassay of MIC testing on MHB, to perform ASTs (Ersoy et al., 2017; Lin et al., 2015). Ersoy et al. conducted a large screen of the resistance pattern of important bacterial pathogens in three different host-mimicking media compared to the pattern in MHB. In as much as a third of the cases, the MICs obtained from host-mimicking media exhibited at least a 4-fold change in MIC. Furthermore, AST performed in host-mimicking media improved the prediction of the appropriate antibiotic therapy in a sepsis murine model. Taken together these results indicate that the standard AST susceptibility testing should be performed in media that better reflect the host milieu. The study conducted by Lin et al. also describes the different effect of antibiotics in a more host-like milieu compared to the standard AST, focusing on the host defense factors that will be present during an infection. They showed that azithromycin in combination with cationic antimicrobial peptides is efficient against multi-drug resistant isolates of *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Acinetobacter baumannii*, bacteria that are usually not sensitive to Azithromycin. In a Commentary to the Lin et al. study, Nizet describes the rather accidental choice of MHB as the gold standard for ASTs and predict the need for media designed to resemble the environmental conditions at the site of infection in predicting better treatment options (Nizet, 2017).

Mecillinam is a β-lactam antibiotic used exclusively to treat uncomplicated Urinary Tract Infections (UTIs) (Gupta et al., 2011; Läkemedelsverket, 2005; Lund and Tybring, 1972; Naber, 2000; Tybring and Melchior, 1975). It was developed in the 1970's and has been used clinically since the early 1980's, mainly in Scandinavia (Naber, 2000; Nicolle, 2000). Due to the rapid development of resistance to many antibiotics used for UTI treatment, such as trimethoprim and fluoroquinolones, mecillinam is now together with nitrofurantoin the first hand choice for treatment of uncomplicated UTIs in Sweden (Kahlmeter, 2002; Kahlmeter et al., 2015; Kahlmeter and Poulsen, 2012; Läkemedelsverket, 2005; Naber et al., 2008). The mutation frequency to Mec^R is very high in laboratory settings, but the frequency of resistance in clinical isolates remains low (Giske, 2015; Kahlmeter et al., 2015). Even though at least 40 genes can confer Mec^R when mutated, only one of them (*cysB*) is involved in Mec^R in clinical *E. coli* isolates from UTI patients (Thulin et al., 2015). The CysB protein is the major positive regulator of the cysteine biosynthesis pathway and turning this pathway off confers Mec^R, but only in growth media that is low in cysteine – if a *cysB* mutant strain is provided with high cysteine levels in laboratory media they phenotypically become mecillinam susceptible (Kredich, 1996; Oppezzo and Antón, 1995; Thulin et al., 2015). However, the mechanism by which *cysB* mutations confer mecillinam resistance remains unclear.

In this study, we show that *E. coli* mutants (both laboratory strains and clinical UTI isolates) that are highly resistant to mecillinam in a standard laboratory medium (MHB) can phenotypically be fully reverted to antibiotic susceptibility when grown in human urine, while still maintaining the resistance mutation. The Mec^R strains reach an MIC of as much as 150 mg/L in MHB, which is well above the mecillinam EUCAST clinical breakpoint of 8 mg/L for resistance. However, when grown in urine the same strains show MICs of mecillinam of 0.25 to 1 mg/L of mecillinam. In addition, the lower the osmolality of urine the more susceptible the bacteria become, implying that the efficiency of mecillinam treatment can be increased, and the risk of resistance evolution decreased, by increased water intake and/or use of diuretics.

2. Materials and Methods

2.1. Bacterial Strains and Media

The strains used in this study are; *E. coli* MG1655 wild type (DA5438), a *cysB* deletion mutant MG1655 (DA28439), and the Mec^R clinical *E. coli* UTI isolates DA14719, DA24682 and DA24686 that are defective in the *cysB* gene. DA14719 and DA24686 were described previously and DA24682 is one of the highly resistant clinical *cysB* mutants (MIC > 256) mentioned in in Thulin et al. 2015 (for more details, see Table 1). The strains were grown in Mueller Hinton (MHB) broth and agar (Difco), in urine (see below), and in Artificial Urine Medium (AUM). When indicated, MHB and urine was supplemented with different concentrations of mecillinam (Sigma-Aldrich) and with 5% sucrose. When appropriate other agar plates were used; 0.5× MHB, 2× MHB, and MHB supplemented with 5% sucrose. AUM agar plates were also used for MIC assays. The AUM plates were prepared as described by Brooks and Keevil, but with 0.4% glucose added (Brooks and Keevil, 1997). When appropriate different concentrations of cysteine were added to the AUM + 0.4% glucose plates.

2.2. Urine Growth Medium

Morning urine was donated by 48 different anonymous healthy male and female volunteers and assigned numbers 1 to 48. Donated urine was kept in 4 °C for maximum five hours, after which pH was measured with pH indicator strips (2.0–9.0) from Merck-Millipore. Subsequently, samples were centrifuged (4500 rpm, 10 min, 4 °C) and sterile filtered (Filtropur BT25, 250 mL, 0.22 μM). Aliquots (45 mL) of the sterile filtered urine were frozen in −20 °C. When used as a growth medium, urine samples were thawed and centrifuged and the supernatant was used as growth medium after a 1 mL aliquot (for metabolome analysis) was separately frozen at −80 °C. Urine A that was used for the original measurements on several different concentrations of Mec and with several different strains was the same described as in Thulin et al. 2015. Urine A was prepared as above, except that it was pooled urine obtained during several mornings from one donor.

For osmolality tests, urine was diluted two- and threefold with sterile H_2O (DEPC-treated and sterile filtered, Sigma Aldrich) or concentrated by drying to a fourth of the volume in a DNA speed Vac and then diluted to half or a third of the original volume with sterile H_2O (as above). The concentrated urine was sterile filtered to ensure removal of any potential contamination during the concentration process.

2.3. MIC Assays

Minimal inhibitory concentrations (MICs) were determined for mecillinam (using MIC test strips from Liofilchem), meropenem (MIC evaluators from Oxoid), ampicillin (Etest strips from bioMerieux) and cefotaxime (Etest strips from bioMerieux). Bacteria were grown overnight in MHB media and then diluted 500-fold in phosphate buffered saline (PBS; 13 mM phosphate, 137 mM NaCl, pH 7.4) before being spread evenly on MHB agar plates (1× MHB, 0.5× MHB, 2× MHB, 1× MHB + 5% sucrose, or AUM + 0.4% glucose + 0, 0.075, 0.15, 0.3, or 0.75 mM cysteine). A MIC test strip, Etest or MIC evaluator was placed on the plates and the results were analysed after ~18 h.

2.4. Growth Measurements

Bacteria were grown in the Bioscreen C Analyser (Oy Growth Curves Ab. Ltd.) using urine or MHB supplemented with different concentrations of mecillinam. Over night cultures of each strain (in each medium)

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