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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.

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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)

Table 1
The strains used in this study and their respective mecillinam MIC on MHB agar (M) with and without 0.75 mM cysteine (C). DA5438 is the *E. coli* MG1655 wild type, DA28439 is a genetically engineered *cysB* deletion mutant and strains DA14719, DA24678 and DA24686 are clinical UTI-isolates carrying different *cysB* mutations.

Strain	Genotype	Origin	Phenotype	Mecillinam MIC	
				M	M + C
DA5438	WT		Cys +	0.125	0.125
DA28439	Δ*cysB*	Laboratory strain (Thulin et al., 2015)	Cys −	32	0.25
DA14719	*cysB* FS after aa 87	Clinical isolate	Cys −	16	0.125
DA24682	*cysB* W89stop	Clinical isolate	Cys −	>256	0.25
DA24686	*cysB* K76stop	Clinical isolate	Cys −	32	0.25

were diluted 2000× in respective medium, with or without mecillinam. In MHB the different *cysB* strains were grown in 0, 10, 19, 37.5, 75, and 150 mg/L Mec and the wild-type strain in 0, 0.25, 0.5, 1, 2, and 4 mg/L Mec. In Urine A all strains were grown in 0, 0.25, 0.5, 1, 2, and 4 mg/L Mec. In urine samples 1 to 48, strains were grown in 0.25 mg/L Mec and if they grew in 0.25 mg/L, also in 1 mg/L. These Mec concentrations were chosen on the basis of the results from a small pilot study. Diluted culture (250μl) was then added to duplicate wells in a Bioscreen C Honeycomb plate. The Bioscreen was run for 8 or 10 h at 37 °C with constant shaking and optical density (OD) measurements at 600 nm every 4 min.

The statistical software R was used to examine the growth curves from the Bioscreen measurements (Team, 2012). The urines were divided into three groups, depending on their growth profile; Urine^{low} (strains grown in these urine samples died at 0.25 mg/L Mec), Urine^{high} (strains grew at 0.25 mg/L, but died at 1 mg/L) and Urine^{int} (urine samples yielding intermediate growth profile).

2.5. Mecillinam Osmolality Stability Test

Microdilution minimal inhibitory concentration assays were performed comparing the efficiency of mecillinam that had been pre-incubated in MHB with or without 5% sucrose with non-treated mecillinam. About 10^6 CFUs of DA5438 wild type strain was inoculated in MHB supplemented with 0, 0.0625, 0.125, 0.25, 0.5, 1, 2 and 4 of the differently treated Mec and then incubated 18 h in 37 °C, after which the results were read. The microdilution MIC assays were tested in duplicates.

2.6. Metabolome Analysis of Urine

Global metabolomic profiles were obtained for 36 urine samples. The metabolome analysis was performed by Metabolon Inc. (Durham, NC, USA). Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy (UPLC-MS/MS) was used. In total 671 different metabolites were identified in the urine samples. See supplement for metabolomics data.

2.7. Statistical Analysis of Metabolome Data

Metabolite levels were compared between two groups of urines; Urine^{low} and Urine^{high}. The amounts of each of the 671 metabolites for the Urine^{low} and Urine^{high} groups were compared using Mann-Whitney tests. As performing such a large number of tests inflates the risk of false discoveries, the *p*-values were adjusted for multiplicity by controlling the false discovery rate (FDR) (Benjamini and Hochberg, 1995).

2.8. Role of the Funding Source

The study was funded by the Swedish Research Council who had no role in study design; in the collection, analysis, and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

3. Results

3.1. cysB Mutants are Resistant to Mecillinam in Laboratory Growth Medium but Susceptible During Growth in Urine

To examine the susceptibility to mecillinam of the wild type (DA5438) and four Mec^R *cysB* mutant strains (DA28439, DA14719, DA24682 and DA24686) in standard laboratory MHB broth and in Urine A, the bacteria were grown at different concentrations of mecillinam. One of the four Mec^R strains was a genetically engineered

Table 2
Growth of different Mec^R strains and wild type *E. coli* in urine (U) and Mueller Hinton Broth (M), both supplemented with the same concentrations of mecillinam. Orange indicates difference in growth between urine and Mueller Hinton at the same mecillinam concentration. (+) indicates growth and (−) indicates no growth. The *cysB* mutations in the clinical strains are either frame shift (FS) or stop codon (SC). Δ*cysB* indicates a deletion of the *cysB* gene.

		Concentration of mecillinam (mg/L)													
		0.25		1		10		19		37.5		75		150	
Strain	**Genotype**	U	M	U	M	U	M	U	M	U	M	U	M	U	M
DA5438	wild type	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DA28439 (laboratory strain)	Δ*cysB*	-	+	-	+	-	+	-	+	-	+	-	+	-	-
DA14719 (clinical isolate)	*cysB* FS	-	+	-	+	-	+	-	+	-	+	-	-	-	-
DA24682 (clinical isolate)	*cysB* SC	-	+	-	+	-	+	-	+	-	+	-	-	-	-
DA24686 (clinical isolate)	*cysB* SC	+	+	-	+	-	+	-	+	-	+	-	+	-	-

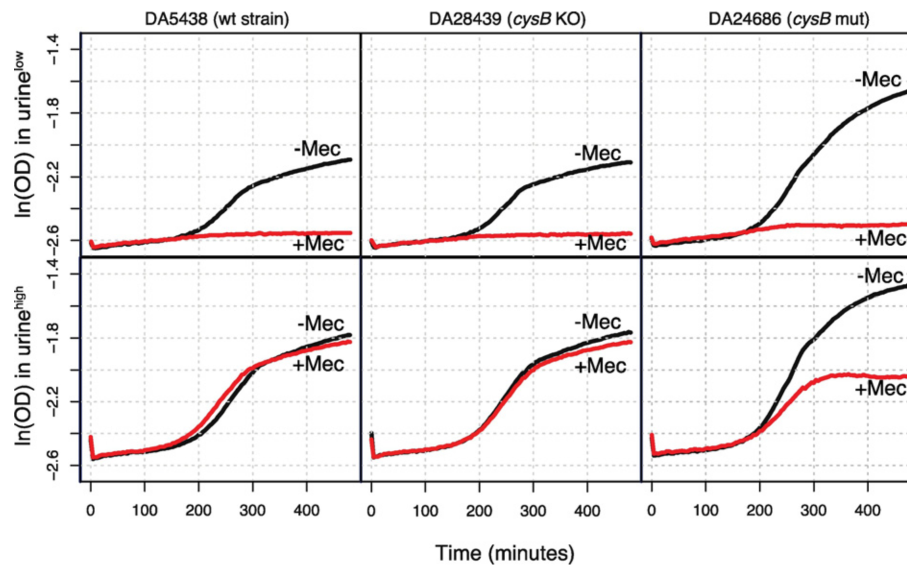

Fig. 1. Examples of growth curves for strains DA5438 (wild type, Mec^S), DA28439 (constructed Δ*cysB* mutant, Mec^R) and DA24686 (clinical isolate with *cysB* mutation, Mec^R) in urine samples from the Urine^{low} group and the Urine^{high} group. Black growth curves were in urine without Mec and red were in urine with 0.25 mg/L Mec. Urine^{low} and Urine^{high} refer to urine in which the MIC of mecillinam was low (0.25 mg/L) and higher (1 mg/L), respectively.

cysB deletion mutant (strain DA28439) whereas the others (strains DA14719, DA24678 and DA24686) were clinical UTI-isolates carrying different *cysB* mutations. The clinical strains were selected on the bases in their difference in mecillinam MIC when measured by MIC test strips, DA14719, DA24686 and DA24682 having 16, 32 and >256 mg/L respectively. The resistance phenotype of all the *cysB* mutant strains is however dependent on cysteine levels, as an addition of 0.75 mM cysteine reduce the MIC of all these strains to wild type levels (Table 1).

When grown in MHB, the *cysB* mutant strains showed high-level mecillinam resistance (MIC = 37.5 to 75 mg/L) in, but all Mec^R strains showed a susceptible phenotype (MIC = 0.25 mg/L), identical to the susceptible strain (Table 2), during growth in urine. Thus, there was a 150- to 300-fold reduction in the MIC of mecillinam during growth in urine. As a control experiment, *cysB* mutant bacteria were recovered after growth in urine and sequenced and tested in MHB medium to confirm that the *cysB* mutation was still present and that the strain maintained a resistant phenotype. In addition, if the recovered strain was again tested in urine it was susceptible. Thus, these tests confirm that it was only the phenotype, and not the genotype, that changed between growth on MHB medium and urine.

3.2. The Level of Mecillinam Susceptibility Varies Between Urine Samples From Different Individuals

To explore in more detail how the resistance phenotype was abrogated when bacteria were grown in urine, we collected morning urine from 48 different healthy male and female donors and used the urine as a growth medium for strains DA5438 (wild type), DA28439 (an engineered *cysB* knock-out mutant) and DA24686 (a clinical Mec^R isolate carrying a *cysB* mutation) in the absence and presence of different concentrations of mecillinam. Growth was examined with all 48 urine samples without mecillinam added and supplemented with 0.25 mg/L or 1 mg/L of mecillinam. As with the previous growth measurements in urine, Mec^R bacteria grown in urine from all 48 individuals were more susceptible to mecillinam than when the same strains were grown in MHB medium. The urine samples were divided into three groups, depending on their growth profile; Urine^{low} (strains grown in these urines died at 0.25 mg/L Mec), Urine^{high} (strains grew at 0.25 mg/L, but growth stopped at 1 mg/L) and Urine^{int} (urine samples with intermediate growth profile). There were 14 urine samples (the Urine^{low} group) in which bacteria was killed at 0.25 mg/L, 13 urine samples (the Urine^{high} group) in which bacteria grew at 0.25 mg/L but was killed at 1 mg/L, and 21 urine samples where growth differed between the three strains or the strains showed poor growth both with and without mecillinam (the Urine^{int} group). In six of the urine samples the bacteria did not grow at all (Supplement, Table 1). Examples of growth curves for the Urine^{low} and Urine^{high} groups are shown in Fig. 1 (all growth curves are found in Supplement Fig. 1 and a list of all urine samples in Supplement, Table 1). In summary, these results demonstrate that for the 42 urine samples in which bacteria grew, the MIC value of mecillinam of the two Mec^R strains (DA28439 and DA24686) was reduced from 75 mg/L (in MHB medium) to 0.25 or 1 mg/L depending on urine sample.

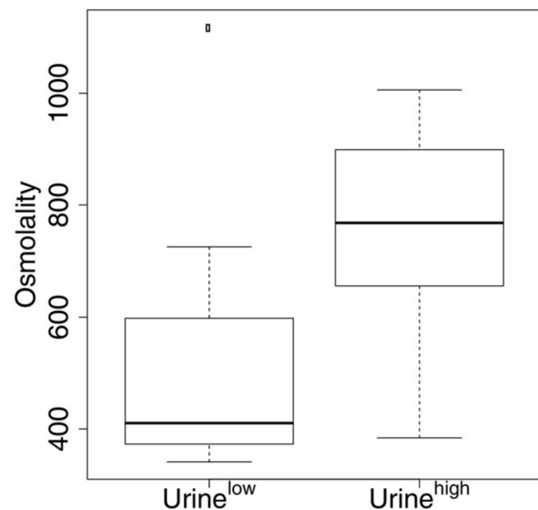

Fig. 2. Boxplot showing the difference in osmolality between urine samples of type Urine^{low} and Urine^{high}, $p = 0.0027$ using Mann-Whitney test. Urine^{low} and Urine^{high} refer to urine in which the MIC of mecillinam was low (0.25 mg/L) and higher (1 mg/L), respectively.

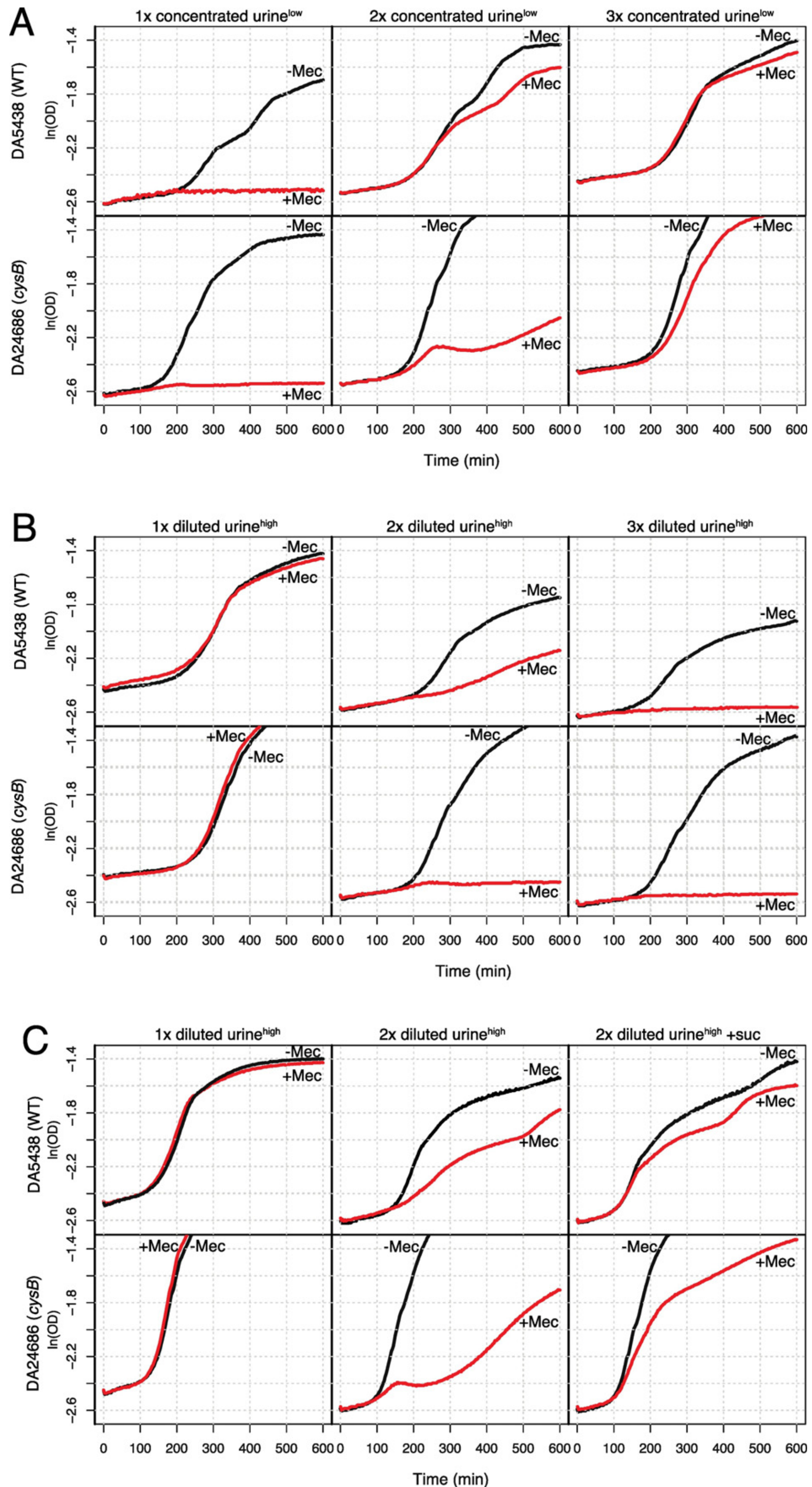

Fig. 3. Growth of strains DA5438 (wild type, Mec^S) and DA24686 (clinical isolate with *cysB* mutation, Mec^R) with and without Mec in (A) concentrated $Urine^{low}$, (B) diluted $Urine^{high}$ and (C) diluted $Urine^{high}$ supplemented with sucrose. Black growth curves were in urine without Mec and red were in urine with 0.25 mg/L Mec.

3.3. Metabolome Analysis Showed That Differences in Osmolality Between Urine Samples Conferred the Differences in Mecillinam Susceptibility

A total of 36 urine samples were analysed for relative metabolite levels: all 14 urine samples from the Urine^{low} group, all 13 from the Urine^{high} group and nine from the Urine^{int} group. In total, 671 metabolites could be identified in the urine (see Supplementary 2) and metabolite levels were compared between two groups of urines, Urine^{low} and Urine^{high}, using Mann-Whitney tests. For 69/671 metabolites, there was a statistical difference in concentration of a metabolite between the groups (using FDR = 0.05), with the Urine^{high} group always containing a significantly higher concentration of the metabolite. We thus hypothesised that the difference between the Urine^{low} group and the Urine^{high} group was osmolality. A Mann-Whitney test showed that there was a two-fold and highly significant difference in osmolality between the Urine^{low} group (low osmolality) and the Urine^{high} group (high osmolality) ($p = 0.0027$) (Fig. 2), suggesting that this difference might cause the difference in mecillinam susceptibility.

3.4. Reduced Osmolality Increased Mecillinam Susceptibility

To confirm that the difference in osmolality was the cause of the difference in susceptibility to mecillinam, we performed several growth tests using strains DA5438 (Mec^S, wild type) and DA24686 (Mec^R clinical UTI isolate). Firstly, we diluted the Urine^{high} two- and threefold to decrease osmolality and as expected the strains became more susceptible (MIC = 0.25 mg/L) in the diluted urine than in the non-diluted urine (Fig. 3A). Secondly, we concentrated the Urine^{low} two- to threefold to increase osmolality and the MIC of mecillinam was increased from 0.25 to 1 mg/L in the concentrated urine (Fig. 3B). Thirdly, we added sucrose to increase the osmolality of the Urine^{low} and the strains showed higher resistance in the sucrose supplemented urine (Fig. 3C). The concentration and dilution (−/+ sucrose) experiments were done with urine samples from two different individuals with the same results. These results demonstrate that the urine osmolality has a direct effect on mecillinam susceptibility. To determine if it is the osmolality of urine or if a change in osmolality in any medium would have a similar effect, we changed the concentration of MHB agar. Dilution of the growth medium reduced MIC of mecillinam whereas concentration of the medium increased MIC of mecillinam (Table 3) for the resistant mutants (DA24686 and DA28439). Furthermore, addition of sucrose to 1xMHB agar increased the MIC of mecillinam for both resistant mutants (Table 3 and Fig. 2C). As a control, we also tested if changes in osmolality affected the MICs of other β-lactams (meropenem, ampicillin and cefotaxime). In contrast to mecillinam these β-lactams showed no or only minor changes in MICs when osmolality was altered (Supplement, Table 2). To decide if the osmolality effect on mecillinam susceptibility was due to differences in stability of Mec at different osmolality, we performed a microdilution MIC assay using Mec that was incubated with and without 5% sucrose and compared this to the efficiency of non-treated Mec. The resulting MICs were 0.5 mg/L regardless of the treatment of the mecillinam used, demonstrating that the osmolality has no effect on Mec-stability.

In conclusion, these results demonstrate that changes in osmolality affects the level of mecillinam resistance and at sufficiently low osmolality a highly resistant strain can become fully susceptible (i.e. identical to wild type susceptibility).

3.5. Reversion of Mecillinam Resistance to Susceptibility in Artificial Urine Medium (AUM) by Cysteine Addition

Since cysteine was present in all of the urine samples we used in this study, we could not assay the impact of cysteine on mecillinam resistance under the relevant in vivo condition (i.e. during growth in urine). To circumvent this problem, we instead used an AUM to show that cysteine addition abrogates mecillinam resistance also in urine (Brooks and Keevil, 1997). To mimic urine with different concentrations of cysteine, MICs of mecillinam were determined on AUM plates containing a concentration gradient between 0 and 0.75 mM of cysteine. As expected, the *cysB* mutants (both laboratory and clinical strains) could not grow on AUM without cysteine (since they are auxotrophic for cysteine biosynthesis). However, as the cysteine concentration in the plates was successively increased, the *cysB* mutants grew and concomitantly the MICs of Mec decreased: from 6 to 12 mg/L at 0.075 mM cysteine down to 0.19 to 0.75 mg/L (similar to wild type level) at 0.75 mM cysteine (Table 5).

4. Discussion

Mutation (inactivation) of the *cysB* gene is the major mechanism of mecillinam resistance in clinical isolates of *E. coli*. This was shown in a previous genetic study of 19 different clinical Mec^R isolates of *E. coli* obtained from UTI patients in Sweden and other European countries, where we found that all strains with a Mec^R phenotype in the EUCAST standard medium MHB had mutations in the *cysB* gene (Thulin et al., 2015). The present study reports two novel findings that are of relevance with regard to the use of mecillinam for treatment of UTIs caused by *E. coli cysB* mutants.

First, to our knowledge this is the first example ever reported, where a genetically stable resistance mutation can be phenotypically reverted to susceptibility when the bacteria are grown in urine. Thus, growth in urine makes mecillinam resistant *E. coli cysB* mutants (both laboratory and clinical strains) susceptible to mecillinam (Table 4). The mechanism of this reversion probably involves the presence of high levels of cysteine and related thiol compounds (e.g. cystine and glutathione) in urine as supported by the following evidence. When grown in a medium with low levels of cysteine, *cysB* mutants are Mec^R, but when the media is supplemented with cysteine (or something that can readily be converted to cysteine such as cystine), *cysB* mutants become susceptible to Mec (Anton, 2000; Oppezzo and Antón, 1995; Thulin et al., 2015). The dependence of the resistance phenotype on low cystein levels is seen for all *cysB* mutants, even the clinical strains with mecillinam MIC test MICs over 256 mg/L which is shown for DA24682 in Table 1, but also for the other highly resistant *cysB* Mec^R strains (data not shown). Worth noticing however, is that the DA24682 strain did not exhibit higher Mec^R than other *cysB* mutants when grown in liquid MHB (Table 2). Increased susceptibility due to cysteine was also observed with artificial urine (Table 5). Furthermore, we showed that all urine samples analysed for metabolites ($n = 36$) contained both cysteine and cystine, and since all the tested *cysB* mutants become more susceptible in all urine samples tested ($n = 42$) these levels are likely high enough to compensate for the inability of *cysB* strains to synthetize their own cysteine. The type of metabolome analysis done here does not provide absolute cysteine concentrations, but it is known from previous studies that 0.3 mM of cysteine or cystine in the growth medium is sufficient to render *cysB* mutant strains Mec^S (Thulin et al., 2015). These levels of cysteine are similar to the mean values of cysteine found in human urine (Pastore et al., 1998; Rafii et al., 2007). In addition, both cysteine and cystine are, independent of individual, always present in human urine as shown in this study and in a previous study (Bouatra

Table 3
MICs of mecillinam of strains DA5438, DA28439 and DA24686 tested with MIC test strips on MHA (M) with different osmolality due to concentration or addition of sucrose.

Strain	Genotype	Phenotype	Mecillinam MIC (mg/L)			
			1× M	0.5× M	2× M	M + suc
DA5438	Wild type	Cys+	0.125	0.032	0.125	0.032
DA28439 (laboratory strain)	Δ*cysB*	Cys−	32	0.05	>256	>256
DA24686 (clinical isolate)	*cysB* K76stop	Cys−	32	4	>256	>256

Table 4
MICs of mecillinam of wild type and *cysB* mutant strains in Mueller Hinton Broth (M) and in urine (U). Supplementation with 0.75 mM cysteine or 5% sucrose is indicated with C and S respectively. Urine^{low} and Urine^{high} refer to urine in which the MIC of mecillinam was low (0.25 mg/L) and high (1 mg/L), respectively. Strains are identified as susceptible (S) or resistant (R) based on the EUCAST clinical breakpoint for mecillinam (8 mg/L).

Strain	Genotype	Phenotype	Mecillinam MIC in mg/L (susceptibility phenotype)						
			M	M + C	U^{low}	U^{high}	conc. U^{low}	dil. U^{high}	U^{low} + S
DA5438	Wild type	Cys+	0.25(S)	0.25(S)	0.25(S)	1(S)	1(S)	0.25(S)	1(S)
DA28439 (laboratory strain)	Δ*cysB*	Cys−	75(R)	0.25(S)	0.25(S)	1(S)	1(S)	0.25(S)	1(S)
DA24686 (clinical isolate)	*cysB* K76stop	Cys−	75(R)	0.25(S)	0.25(S)	1(S)	1(S)	0.25(S)	1(S)

et al., 2013). Overall, these findings support the notion that the cysteine present in urine confers some degree of mecillinam susceptibility as compared to a laboratory medium such as MHB.

Second, osmolality of urine also alters susceptibility such that dilution of urine or MHB medium results in increased susceptibility, whereas concentrating the urine or MHB medium or adding 5% sucrose results in an increased mecillinam resistance. Osmolality of growth media has previously been shown to have an effect on susceptibility to β-lactam antibiotics of bacteria. For example, Greenwood et al. showed that low osmolality medium increased early lysis of ampicillin treated *E. coli* compared to higher osmolality medium and that this effect was also seen in the specific case of mecillinam (Greenwood and O'Grady, 1973, 1972). However, when we examined the effect of osmolality on the MIC of meropenem, ampicillin and cefotaxime, we saw only minor differences on media with different osmolalities (Supplement, Table 2A, B and C), compared to the very large effects seen on mecillinam MIC (Table 3). An explanation for the osmolality effect being stronger in the case of mecillinam can be the effect of mecillinam is the formation of spherical cells with increased volume (Greenwood and O'Grady, 1973). Such cells are likely to be more vulnerable to the osmotic pressure of the medium.

These findings have several important implications that are of clinical relevance. First, they imply that to obtain clinically relevant resistance determinations, testing of mecillinam resistance in the clinical microbiology laboratory ought to be done under conditions that mimics urine containing cysteine at the levels typically present in urine (e.g. artificial urine medium with cysteine added) rather than standard media such as MHB (Mueller-Hinton). A second implication is that the clinical UTI strains that are being identified as Mec^R by the standard testing done in clinical laboratories (MIC tests or disc diffusion on MHB agar) may not be resistant during growth in the bladder of a patient and therefore still treatable with mecillinam. This finding illustrates a key problem in clinical bacteriology, namely how can we be sure that a strain that is classified as resistant in the laboratory is in fact still phenotypically resistant and non-treatable also during growth in the patient. This problem has recently been addressed and more evidence is accumulating suggesting a shift to use more host-like media when performing ASTs (Ersoy et al., 2017; Lin et al., 2015; Nizet, 2017). There also are other methods of identifying resistance than measuring MIC (or zone diameter), both phenotypic and genotypic (Balouiri et al., 2016; Turnidge and Paterson, 2007). Which would be the appropriate test and which media to use, would have to be decided on the antibiotic, infection-site and pathogen, and will of course be both time and resource consuming. But as the resistance problem is only increasing, the necessity for more fine-tuned measurements of resistance will only grow stronger, and using actual body fluids, or media more like them is likely to be a part of this solution. In this paper, we showed that Artificial Urine Medium supplemented with cysteine yielded the same results as using actual urine, but this was based on the information we already had on the resistant strains. If the resistance mechanism of a strain is unknown, which will be the case in a majority of patient samples, probably the best choice would be to perform ASTs in media as similar to real body fluids as possible.

An example of when more detailed prediction of treatment outcome is already in use is the site-specific breakpoints issued for pneumonia caused by penicillin resistant *Streptococcus pneumoniae*, which is still treatable with penicillin due to the fact that drug concentrations in lung alveoli reaches high enough levels to kill even resistant strains (Chiou, 2006; Peterson, 2006). In this case, EUCAST and CLSI have based their clinical breakpoints and treatment recommendations of at what penicillin dosage, *S. pneumoniae* strains causing pneumonia are considered susceptible, depending on their MIC. Finally, since mecillinam resistance is conditional it might be reversible by altered patient behavior. Thus, reducing urine osmolality, for example, by increased water intake or use of diuretics would be expected to increase the susceptibility of Mec^R strains. Importantly, increased water intake will have two beneficial effects: first, an increased rate of micturition will increase the demand on the resistant sub-population to grow sufficiently fast to allow its maintenance in the infected host and second by conferring mecillinam susceptibility as described here (Nilsson et al., 2003; Sandegren et al., 2008; Thulin et al., 2015). Also, maintaining high cysteine levels in urine (potentially by eating cysteine rich food) would be expected to increase the susceptibility of Mec^R strains. Further clinical studies are needed to test these ideas.

Contributors

ET and DIA designed the study and DIA obtained project funding. ET collected samples and performed the experiments. MT was responsible for statistical analysis and programming of the R scripts used by ET and MT for analysis of growth curves. ET and DIA drafted the report. All

Table 5
MICs of mecillinam for wild type and *cysB* mutant strains in AUM + glucose at different concentrations of cysteine. DA5438 is the *E. coli* MG1655 wild type, DA28439 has a genetically constructed deletion of *cysB* and DA24686 is a clinical strain carrying a *cysB* K76stop mutation (SC).

Strain	Genotype	Phenotype	Mecillinam MIC (mg/L) at different concentrations of cysteine (mM)				
			0 mM	0.075 mM	0.15 mM	0.3 mM	0.75 mM
DA5438	Wild type	Cys+	0.38	0.38	0.38	0.38	0.38
DA28439 (laboratory strain)	Δ*cysB*	Cys-	[a]	8	4	2	0.75
DA24686 (clinical isolate)	*cysB* SC	Cys-	[a]	12	8	4	0.19

[a] The *cysB* mutant strains do not grow on AUM without cysteine since they are cysteine auxotrophs.

authors critically read and commented on draft versions of the report, and approved the final version.

Declaration of Interests

DIA is a consultant for Bactiguard, Prebona and Utility.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2017.08.021.

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