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Cryptic silver resistance is prevalent and readily activated in certain Gram-negative pathogens

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Running title: Cryptic silver resistance in Gram-negative bacteria

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Abstract

Objectives: To assess the prevalence of cryptic silver (Ag^+) resistance amongst clinical isolates of Gram-negative bacteria, and to examine how overt Ag^+ resistance becomes activated in such strains.

Methods: Established methods were used to determine the susceptibility of 444 recent clinical isolates to Ag^+, and to evaluate the potential for overt Ag^+ resistance to emerge from these isolates by spontaneous mutation. The genetic basis for Ag^+ resistance was investigated using PCR amplification and DNA sequencing.

Results: None of the isolates tested displayed overt Ag^+ resistance. However, upon silver challenge, high-level Ag^+ resistance (silver nitrate MIC >128 mg/L) was selected at high frequency (10^{-7} to 10^{-8}) in ~76% isolates of *Enterobacter* spp., ~58% isolates of *Klebsiella* spp., and ~0.7% isolates of *E. coli.* All strains in which Ag^+ resistance could be selected harboured the *sil* operon, with resistance in each case apparently resulting from activation of this system as a consequence of a single missense mutation in *silS*. By contrast, Ag^+ resistance could not be selected in isolates lacking *sil*, which included all tested representatives of *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Proteus* spp and *Citrobacter* spp.

Conclusions: Whilst overt Ag^+ resistance in Gram-negative pathogens is uncommon, cryptic Ag^+ resistance pertaining to the *sil* operon is prevalent and readily activated in particular genera (*Enterobacter* and *Klebsiella*).

Introduction

The silver cation (Ag^+) has a long history of use as an antimicrobial agent, and continues to be deployed extensively in the healthcare setting for the prevention and treatment of bacterial infection. [1, 2] In view of this extensive use, concerns have been raised over the potential for Ag^+ resistance to emerge in bacteria of clinical relevance and to thereby compromise its therapeutic utility. [3, 4] Although there is no evidence to date of Ag^+ resistance in Gram-positive pathogens such as *Staphylococcus aureus*, [5] several studies have reported a low prevalence of Ag^+ resistance in Gram-negative pathogens. [6-9] In the latter, resistance to Ag^+ results from expression of the Sil system, which mediates resistance through a combination of Ag^+ sequestration in the periplasm (by the SilE protein) and efflux (via the SilCFBA transporter). [4, 10] Here we report that, whilst resistance to Ag^+ is indeed uncommon amongst Gram-negative bacteria, a substantial proportion of some Gram-negative species harbour a cryptic *sil* operon that can be activated by mutation to yield overt resistance.

Our decision to investigate the phenomenon of cryptic Ag^+ resistance was prompted by observations that collectively suggest that the *sil* operon is not ordinarily expressed in the pathogens that harbour it, and that derepression of expression of this system is required for overt Ag^+ resistance to manifest. In particular, a number of reports have noted the presence of genes encoding Sil-system components in Gram-negative species that are nonetheless susceptible to Ag^+. [4, 9, 11, 12] For example, in a recent study we described individual strains of *Klebsiella pneumoniae* and *Enterobacter cloacae* that carry the *sil* operon but do not exhibit phenotypic resistance. [4] In these strains, overt resistance could be readily activated via a single missense mutation in *silS*, [4] a gene which encodes a putative sensor kinase that positively regulates expression of the *sil* operon. [10] Furthermore, amongst clinical isolates exhibiting overt resistance to Ag^+, there is evidence to suggest that resistance is the result of activation of a once-cryptic Sil system; expression of the *sil* operon on the first reported Ag^+-resistance plasmid (pMG101) was shown to be constitutively high compared with that found on closely-related plasmids that do not confer Ag^+- resistance. [13]

To date, there have been no surveys into the prevalence of cryptic Ag^+ resistance in clinical isolates. Using a recent collection of Gram-negative pathogens isolated from patients from the UK and abroad, we confirm here that overt Ag^+ resistance is not common amongst this

group of bacteria. We also establish that cryptic resistance is prevalent in some genera; the majority of *Enterobacter* spp. and *Klebsiella* spp. tested contained a cryptic Ag^+ resistance determinant, and these strains were able to evolve high-level Ag^+ resistance via a single mutational event.

Materials and Methods

Bacteria, culture conditions and susceptibility testing

Bacterial isolates used in this study (*n*=444; Table 1) were recovered from patients in the UK, Ireland and the USA between 2012 and 2015. Bacteria were routinely cultured at 37^{o}C using Mueller Hinton agar (MHA) or broth (MHB). Susceptibility of bacteria to Ag^{+} (in the form of silver nitrate [$AgNO_3$; Sigma Aldrich, Dorset, UK]) was determined by agar dilution using a validated method [5] based on the CLSI guidelines for antibacterial drug susceptibility testing. [14]

Selection and characterization of Ag^{+} resistance

Selection of spontaneous mutants resistant to Ag^{+} was carried out essentially as described, [4, 15] and involved plating saturated cultures (>10^9 cfu/ml) onto MHA containing 128 mg $AgNO_3$/L. The frequency with which mutants resistant to Ag^{+} arose was determined using standard methodology. [15]

Detection of genes encoding the Sil system was achieved by colony PCR using GoTaq® Green mastermix (Promega, Southampton UK) and oligonucleotide primers directed at highly conserved regions within the *sil* operon that flank the *silS* gene (*5'*-AGCGACTCCGCGCTAAAATA and *5'*-GGCTTCTGTTTGCTGCATGA [Eurofins Genomics, Ebersberg, Germany]). The resultant PCR amplicons were purified using the QiaQuick kit (Qiagen, Hilden, Germany) and subjected to DNA sequence determination (GeneWiz, Essex UK) using the amplification primers above and two internal sequencing primers (*5'*-CTCCATTACCTCGATGCGCT and *5'*-GTTCCTGGCACAGGCAGATA).

Results and discussion

From diverse sources, we assembled a collection of 444 clinical isolates comprising the most significant Gram-negative genera associated with skin and soft tissue infection (Table 1), and tested their susceptibility to Ag^+. All isolates were found to be susceptible to 1-8 mg/L $AgNO_3$ (MIC_{50} of 4 mg/L, MIC_{90} of 8 mg/L). These values are similar to those seen for Ag^+-susceptible laboratory strains of *E. coli* (MICs of 4-8 mg/L [4]) and are substantially lower than those observed for known Ag^+-resistant strains (MICs of >128 mg/L [4]). Consequently, we consider all of the isolates in this study to be Ag^+ susceptible, and in the absence of clinical breakpoints propose an epidemiological cut-off (ECOFF) [16] of ≤8 mg/L, when using the susceptibility testing methodology employed here, to distinguish Gram-negative strains whose response to Ag^+ is 'wild-type' from those that express an Ag^+ resistance mechanism. Our data corroborate previous reports that overt Ag^+ resistance is rare amongst Gram-negative pathogens. [6, 9]

To detect cryptic Ag^+ resistance, we screened all 444 isolates for their ability to yield resistant colonies upon Ag^+ challenge. Plating of saturated cultures onto agar containing $AgNO_3$ at 128 mg/L selected mutants exhibiting Ag^+ resistance (MIC >128 mg/L) in ~76% isolates of *Enterobacter* spp., ~58% isolates of *Klebsiella* spp., and ~0.7% isolates of *E. coli* (Table 1). The frequency with which Ag^+-resistant mutants arose was subsequently measured in a subset of these strains (*n*=60). Mutation frequencies to Ag^+ resistance in all 60 isolates were similar (ranging from ~1.5×10^{-8} to ~5.2×10^{-7}), and sufficiently high to imply that resistance was the result of a single mutational event in each case. By contrast, resistance to Ag^+ could not be selected under these conditions in any of the other genera tested (Table 1).

To determine the genetic basis for Ag^+ resistance in the mutants recovered, we proceeded on the basis that the Ag^+ resistance phenotype in these strains was likely the result of mutational activation of a cryptic Sil system, and screened for the presence of the *sil* operon using PCR to detect *silS*. In all 119 isolates in which spontaneous resistance to Ag^+ could be selected, *silS* was detected; by contrast, *silS* was not found in a cross-section of isolates (*n*=30) from which Ag^+ resistant mutants were not recovered. DNA sequence determination of the entire *silS* gene from all 119 *silS*$^+$ isolates, and from a single Ag^+ resistant mutant recovered from each isolate, revealed a single missense mutation in each resistant mutant by comparison with the Ag^+-sensitive parental strain (*data not shown*). Consequently, it appears that Ag^+ resistance

in all mutants selected is attributable to activation of the Sil system as a result of mutational change in *silS*.

The SilS protein is the sensor histidine kinase (SHK) of a two-component regulatory system that acts to positively regulate the *sil* operon via phosphorylation of the transcription factor, SilR. [17] Though SilS is itself not well studied, SHK proteins as a class have been extensively investigated, offering the opportunity to understand mutational gain of function in the SilS protein by analogy. We employed Pfam [18] searches to predict the location and extent in SilS of functional domains that are common to SHK proteins, and used this information to produce a schematic of SilS onto which all of the substitutions identified in this study were mapped (Figure 1). This analysis revealed that the substitutions leading to activation of SilS are clustered within three functionally-distinct regions; the transmembrane domains, the dimerization/histidine phosphotransfer (Dhp) domain and the catalytic/ATP-binding (CA) domain (Figure 1). Substitutions identified in the latter two domains are located at similar positions to those found in mutants of the SHKs EnvZ and PhoB that exhibit constitutive activation as a consequence of being unable to dephosphorylate their cognate response regulator, [19] suggesting that this is also the case for these mutant SilS proteins. By contrast, the substitutions found in the transmembrane regions adjacent to the sensory domain may prompt constitutive activation of the Sil system by uncoupling signalling from sensing. [20]

In summary, within a large collection of recent Gram-negative clinical isolates, no overt resistance to Ag^+ was detected. Nevertheless, amongst *Klebsiella* and *Enterobacter spp*., high-level (MIC >128 mg/L) Ag^+ resistance was readily selected in the majority of isolates, with resistance arising in all cases apparently as a consequence of a single mutation in *silS* that led to activation of the Sil system. The high frequencies (*c.* 10^{-8}) with which Ag^+ resistance arose in these isolates is comparable to that seen for antibiotics that act upon a single cellular target, agents that are generally considered unsuitable for monotherapy owing to resistance liabilities and the consequent likelihood that resistance will arise during treatment. [21] Thus, we consider that cryptic resistance has the potential to compromise the efficacy of Ag^+-based therapeutics, and suggest that it would be of benefit for clinicians to screen patients for the presence of strains harbouring cryptic Ag^+ resistance determinants prior to deployment of such agents. We recommend the use of the simple screening approach for cryptic Ag^+ resistance that we have employed here, involving the plating of saturated cultures of isolates

onto agar containing suprainhibitory concentrations of Ag^+, and noting the growth of resistant colonies following overnight incubation.

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Transparency declaration

All authors: None to declare.

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Table 1. **Prevalence of cryptic Ag^+ resistance amongst the isolates analysed in this study.**

Isolate type	Number of isolates tested	Number of isolates in which Ag^+ resistance could be selected (%)
Acinetobacter spp.	27	0
Citrobacter spp.	5	0
Enterobacter spp.	75	57 (76%)
Escherichia coli	135	1 (0.7%)
Klebsiella spp.	105	61 (58%)
Proteus spp.	6	0
Pseudomonas aeruginosa	91	0

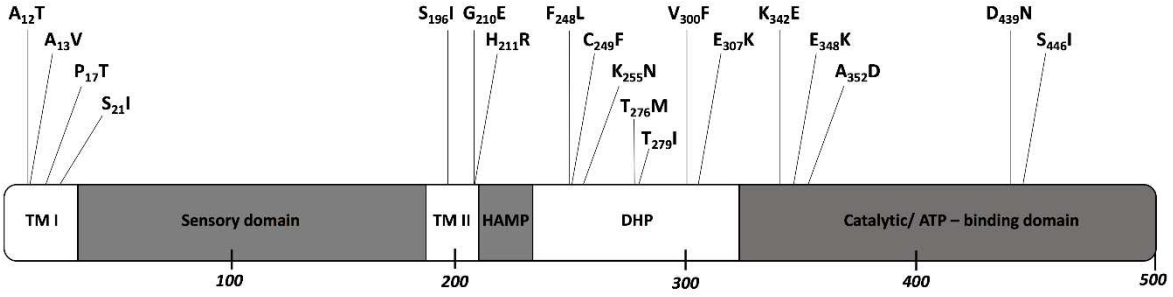

Figure 1. Schematic of the SilS protein showing the predicted domain architecture by comparison with other sensor histidine kinase proteins, and indicating the sites of amino acid substitutions that activate the cryptic Sil system. The amino acid substitutions shown were all detected in single mutants, with the exception of $A_{13}V$ and $G_{210}E$ that were found in mutants selected from two and three independent isolates, respectively. TM: transmembrane domain; HAMP: histidine kinase/adenylyl cyclase/methyl-accepting/phosphatase domain; DHp: dimerization and histidine phosphotransfer; CA: catalytic and ATP-binding domain.