New insights into the regulatory pathways associated with the activation of the stringent response in bacterial resistance to the PBP-2 targeted antibiotics, mecillinam and OP0595/RG6080

M. Doumith[1*], S. Mushtaq[1], DM. Livermore[1,2] and N. Woodford[1].

[1]National Infection Service, Public Health England, NW9 5EQ London United Kingdom; [2]Norwich Medical School, University of East Anglia, Norwich, NR4 7TJ, United Kingdom

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Corresponding author

Michel Doumith
National Infection Service, Public Health England, NW9 5EQ London United Kingdom
Email: Michel.Doumith@phe.gov.uk
Tel: +44(0)20 8327 6834

BACKGROUND*:* The diazabicyclooctane β-lactamase inhibitor OP0595 (RG6080) also acts as an antibiotic, targeting penicillin-binding protein 2 (PBP2) in Enterobacteriaceae but this activity is vulnerable to mutational resistance. We used whole genome sequencing (WGS) to investigate the basis of this resistance. METHODS: Twenty OP0595-selected mutants, comprising four derived from each of five different *Escherichia coli* strains, were sequenced on Illumina HiSeq. Reads from each mutant were mapped to the assembled genome of the corresponding parent. A variant-calling file generated with Samtools was parsed to determine genetic alterations. RESULTS: Besides OP0595, the mutants consistently showed decreased susceptibility to mecillinam, which likewise targets PBP2, and grew as stable round forms in the presence of subinhibitory concentrations of OP0595. Among the 20 mutants, 18 had alterations in genes encoding tRNA synthase and modification functions liable to induce expression of the RpoS sigma factor through activation of the stringent response or had mutations suppressing inactivators of RpoS or the stringent response signal-degrading enzyme, SpoT. TolB was inactivated in one mutant: this activates RscBC regulation and was previously associated with mecillinam resistance. The mechanism of resistance remained unidentified in one mutant. Both the RpoS and RscBC systems regulate genes of cell division, including *ftsAQZ* that can compensate for loss or inhibition of PBP2, allowing survival of the challenged bacteria as stable round forms, as seen. CONCLUSIONS: WGS identified the global stringent response signal, entailing induction of RpoS, as the main mediator of mutational resistance to OP0595 in *E. coli*.

Introduction

Production of β-lactamases is the prevalent mode of resistance to β-lactam antibiotics in Gram-negative bacteria. To counter this, several new β-lactamase inhibitors are under clinical development, including several diazabicyclooctanes, such as OP0595 (RG6080).[1]

OP0595 inhibits Class A and C serine β-lactamases and, also acts as an antibiotic, targeting penicillin-binding protein 2 (PBP2) of Enterobacteriaceae as with mecillinam. Furthermore, and independently of β-lactamase inhibition, OP0595 acts as an 'enhancer,' synergising β-lactams that bind to PBP-3.[1] Its antimicrobial activity is vulnerable to high-frequency mutational resistance and we used WGS to investigate its genetic basis.[1, 2]

Materials and methods

Selection and characterisation of OP0595-resistant mutants

OP0595-resistant mutants from five different *E. coli* strains were selected by applying overnight broth culture on Muller-Hinton agar containing OP0595 at 16 mg/L.[2] Parent and mutant cell shapes were investigated under microscopy after 2h incubation in broth supplemented with OP0595 at multiples of MICs for the parent strains. Images were taken after bacterial staining with 1.5% phosphotungstic acid, using a JEM-1400 transmission electron microscope (JEOL, Peabody, MA, USA) fitted with an AMTX XR60 camera. Susceptibility testing was performed by agar dilutions according to BSAC guidelines.[3]

Sequencing and bioinformatics

Parent and mutant DNA were extracted on the QIAsymphony automated platform (QIAGEN, Hilden, Germany) used according to the manufacturer's instruction. Paired-end reads of 2 x 100 nucleotides with over 30 times depth of coverage were generated for each sequenced DNA on a HiSeq Illumina instrument using the Nextera XT DNA sample preparation kit (Illumina, San Diego, CA, USA). Reads

were trimmed to remove low-quality nucleotides using Trimmomatic 0.32 (http://www.usadellab.org/cms/?page=trimmomatic), specifying a sliding window of 4 with average Phred quality of 30 and 50 as the minimum read length to be conserved. Trimmed reads for parents and mutants were assembled into contigs with VelvetOptimiser 2.1.9 software (http://bioinformatics.net.au/software.velvetoptimiser.shtml), using *k-mer* values from 55 to 75. Only contigs ≥ 300 bp were used in further analysis. Reads from the four mutants of each set were individually mapped to the assembled contigs of the corresponding parent, using Bowtie2 (http://bowtie-bio.sourceforge.net/bowtie2) in a global alignment mode to generate a sequence alignment/map (SAM) file, which was used to generate a variant-calling file (VCF) using the Samtools 0.1.18 algorithm (http://samtools.sourceforge.net) with default settings. Base polymorphisms and small indels were detected using an in-house Python script which parsed the VCF file line-by-line to determine the base calls at each nucleotide position, with filtering based on the read coverage (≥ 5 reads), frequency of polymorphic bases (≥ 80 %) and the overall quality of the variant call (base mapping ≥ 25 Phred score). Potential large deletions or insertions were checked in the VCF by filtering for the read coverage (≤ 2) and the frequency of the read- start and end information (≥ 50 %). All detected base polymorphisms were manually confirmed on Tablet 1.14 (https://ics.hutton.ac.uk/tablet/) and suspect genomes carrying large genetic alterations were visualized using Mauve 2.3.1 software (http://darlinglab.org/mauve/mauve.html). The Illumina sequences generated in this study are deposited and available in the European Nucleotide Archive (ENA) under the study accession number PRJEB12745 (http://www.ebi.ac.uk/ena/data).

Results and Discussion

MICs of OP0595 for the twenty mutants exceeded 32 mg/L, compared with 0.5-1 mg/l for their susceptible parent strains.[2] The mutants exhibited at least an eight fold increase in resistance to mecillinam, which also solely targets PBP2, whereas MIC shifts of β-lactam antibiotics targeting other PBPs were variable and lacked any consistent trend.[2] To elucidate these resistance traits, parent and

mutant genomes were sequenced and various alterations were identified in multiple regions of the chromosome. These ranged from single nucleotide substitutions to deletions or insertions of 2 to 3767 nucleotides. Based on the annotation of *E. coli* published genomes, we located alterations to coding fractions of the genome that were inferred to result in amino acid changes or loss in 19 of the 20 mutants, including eight cases where replacements generated a premature translation-termination codon (Table 1). Only one mutant (EC-4 M2) had an alteration in a non-coding intergenic region, involving an insertion sequence 225 bp upstream from the transcription start of the global two-component system *arcA* gene (Table 1).

Fifteen different altered genes were detected among the twenty OP0595-resistant mutants. These did not include *pbp2*, which encodes the OP0595 target, PBP2. Rather, seven genes, namely *lysS*, *alaS*, *aspS*, *ileS*, *cca, hemL* and *mnmA*, variously altered in 10/20 mutants, encoded aminoacyl tRNA synthesis and modification functions (Table 1). Alterations in the coding sequences of *alaS*, *aspS* and other (e.g.. *arg*S*, thrS, leuS* and *gltX*) tRNA synthetase genes have previously been associated with mecillinam resistance, and are known to result in increased intracellular levels of the stringent response signal mediator guanosine-3',5'-bisdiphosphate (ppGpp).[4-6] The stringent response is a widespread global regulatory system, activated in response to various stresses. Production of ppGpp depends on the ribosome-associated protein RelA, which is activated under amino acid limitation, and when uncharged tRNAs bind the ribosomal A site.[7, 8] Degradation of ppGpp, upon return of favourable conditions, is catalyzed by SpoT, a bifunctional enzyme that can also synthesize ppGpp in response to carbon, fatty acid and iron limitation, although less efficiently than RelA.[7, 8] ppGpp primarily regulates gene transcription and is required for the expression of the sigma factor RpoS, which is known to regulate multiple genes and, in particular, those associated with cell division at stationary phase, including the *ftsAQZ* operon, activation of which may be the effector mechanism for resistance to PBP2-targeted agents.[9, 10] The alterations identified in genes encoding aminoacyl tRNA synthesis and modification functions in these 10 OP0595-resistant mutants (Table 1) would be expected to decrease

111 the aminoacyl tRNA levels in the cell, mimicking the amino acid starvation stress conditions that
112 activate RelA to produce ppGpp.

113 Five of the remaining 10 mutants had alterations in the coding sequence or potential regulatory region
114 of the global two-component systems *arcA* or the cytochrome D-ubiquinol oxidase subunit *cydA* (Table
115 1). Inactivation of either *arcA* or *cydA* has been shown to increase the expression of the sigma
116 regulator factor *rpoS*.[11, 12] Of the final five mutants, one had an alteration in the ppGpp degrading
117 enzyme SpoT and one had the 50S rRNA methyltransferase Rlm inactivated, together with a possibly
118 insignificant mutation in *ribE*. Mutations in rRNA methyltransferase result in a slow-growing
119 phenotype, which also may induce the stringent response.[13] Another mutant had alterations in the
120 RNA polymerase subunit RpoC, which interacts with RpoS and, although the mechanism of linkage is
121 uncertain, alteration to the second subunit of the RNA polymerase, RpoB, was previously associated
122 with mecillinam resistance.[14] Another mutant had inactivation of TolB, a periplasmic component of
123 the Tol-Pal system involved in maintaining outer membrane integrity.[15] Release of periplasmic
124 components into the extracellular medium of *tol-pal* mutants leads to osmosensitivity and activates
125 the sensor protein RcsC which, with RscB, regulates expression of *ftsAQZ* independently from the RpoS
126 pathway.[16-20] Alterations of RcsBC regulation have been associated with mecillinam resistance in *E.*
127 *coli* mutants.[21] The origin of resistance remained unclear in one mutant (EC-3 M-4), which had
128 alteration only in *bcsC*, which encodes a cellulose synthase

129 Induction of either the RpoS or RscCB regulatory pathways stimulates expression of FtsZ, the
130 possible mediator. Of the six promoters identified upstream of *ftsZ* in *E. coli*, *ftsQ1p* is recognized by
131 RpoS whereas *ftsA1p* is stimulated by the two-component system RcsBC.[16, 22-25] FtsZ is widely
132 conserved among prokaryotes and shares a common ancestor with eukaryotic tubulin.[26] It can
133 modulate membrane plasticity, and overexpression in *E. coli* has been reported to allow stable growth
134 as round-cell shape to compensate PBP2 loss.[27] All the OP0595-resistant mutants exhibited spherical
135 forms after two hours incubation in broth supplemented with sub-MICs of OP0595 (Figure 1), an

observation in keeping with previous data for mecillinam and with the view that resistance to these agents entails compensation for inhibition of PBP2, not modification or shielding of this target.[28]

Combining whole genome sequencing with published experimental data of altered genes in OP0595-resistant mutants is sufficient to elucidate the underlying molecular mechanisms (Figure 2). In brief, the main mechanism of resistance to OP0595 is activation of RpoS either: (i) through stimulation of the stress stringent response, or (ii) by inactivation of RpoS suppressors, such as AcrAB and CydA (Figure 2). Activation of the RcsCB regulation system, previously identified in mecillinam-resistant mutants, also can potentially lead to OP0595 resistance.[5, 21] Both RpoS and RcsC regulate genes encoding cell division functions, specifically *ftsAQZ*, that can compensate for PBP2 loss or inhibition, allowing survival of the challenged bacteria as stable round forms. This hypothesis is in keeping with the observed morphological effects and with the fact that OP0595 continues to act as a β-lactamase inhibitor and as a β-lactamase-inhibition independent synergist ('enhancer') of PBP3-targeted antibiotics against Enterobacteriaceae that are resistant to its direct antibacterial activity.[1, 2]

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DML: Advisory Boards or ad hoc consultancy – Accelerate, Achaogen, Adenium, Alere, Allecra, Altermune, Astellas, AstraZeneca, Auspherix, Basilea, Bayer, BioVersys, Cubist, Curetis, Cycle, Discuva, Forest, GSK, Meiji, Pfizer, Roche, Shionogi, Tetraphase, VenatoRx, Wockhardt; Paid lectures – AOP Orphan, Astellas, AstraZeneca, Bruker, Curetis, Merck, Pfizer, Leo; shareholdings in– Dechra, GSK, Merck, Perkin Elmer, Pfizer amounting to <10% of portfolio value. The remaining authors have none to declare

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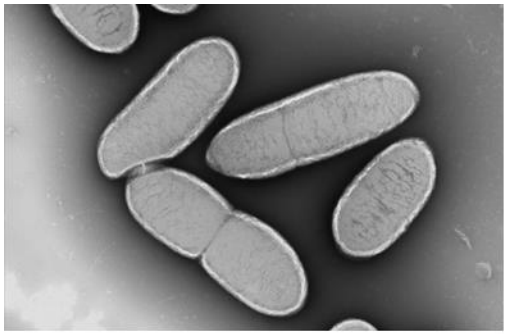
Table 1. Gene alterations associated with OP0595 resistance from this study.

Isolate		Alterations		Genes	Functions
		nucleotides	**Amino acids**		
EC-1	M-1	T=>C	R337C	*rpoC*	DNA-directed RNA polymerase
	M-2	del-TGTTGCG	L136*	***cca***	tRNA nucleotidyl transferase
	M-3	A=>G	G144S	***hemL***	glutamate-1-semialdehyde-2,1-aminomutase
	M-4	A=>G	G474S	***lysS***	lysine tRNA ligase
EC-2	M-1	A=>C	V54G	*ribE*	riboflavin synthase beta chain
	M-1	G=>A	W91*	*rlm*	50S rRNA methyltransferase
	M-2	C=>T	T36A	***alaS***	alanyl-tRNA synthetase
	M-3	G=>A	Q192*	*tolB*	periplasmic protein, TonB-independent uptake of group A colicins
	M-4	G=>A	Q371*	*cydA*	cytochrome d ubiquinol oxidase, subunit I
EC-3	M-1	del AG	L351*	*arcB*	aerobic respiration control sensor protein
	M-2	C=>A	E335*	***mnmA***	tRNA (Gln, Lys, Glu) 5-methylaminomethyl-2-thiouridylase methyltransferase
	M-3	del 3767 bp		***lysS***	lysine tRNA ligase - isopentenyl-diphosphate isomerase$^{\$}$- hypothetical$^{\$}$- purine permease$^{\$}$
	M-4	C=>G	P1019A	*bcsC*	cellulose synthase subunit
EC-4	M-1	C=>T	P555S	***aspS***	aspartyl-tRNA synthetase
	M-2	IS insertion		intergenic	225-bp upstream *arcA* - potential regulatory region
	M-3	A=>T	V74E	*spoT*	guanosine-3',5'-bis(diphosphate)
	M-4	G=>T	E329*	*cydA*	cytochrome d ubiquinol oxidase, subunit I
EC-5	M-1	T=>C	F197L	***aspS***	aspartyl-tRNA synthetase
	M-2	Ins-ACGCGTATT	133-LRV	***cca***	tRNA nucleotidyl transferase
	M-3	Ins-TAC	153Y	***ileS***	isoleucyl-tRNA ligase
	M-4	del-TGATGTCC	I152*	*arcA*	DNA-binding response regulator in two-component regulatory system with ArcB

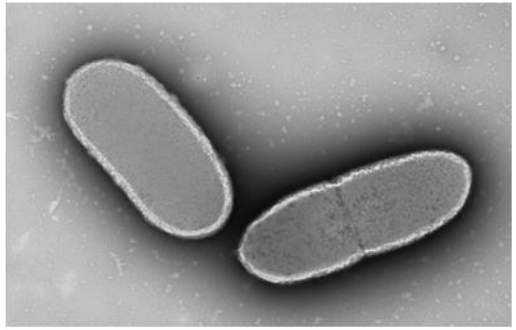
All annotations were in relation to the published *E. coli* MG1665 genome (GenBank: U00096).

Genes shown in **bold** font encode synthesis or modification of amino-acyl tRNAs. (*) indicated stop codon ($) genes in the deleted DNA fragment that are unlikely to be associated with resistance.

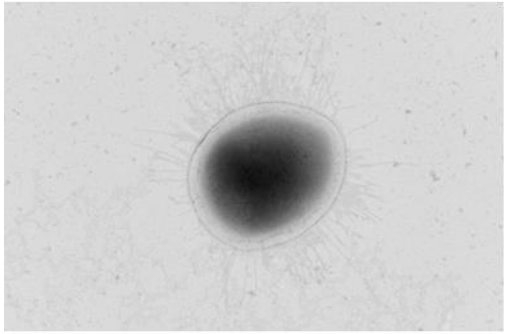
Figure 1. Parent and their OP0595-selected mutants after two hours incubation with OP0595 at 2 x MIC for the parent strains (1-2 mg/L); these concentrations are $\leq 1/16^{th}$ the MIC for the mutants. The distinction is that the mutants conserve their round shapes in the absence of OP0595 and can survive the inhibition of PBP2, whereas their parents cannot.



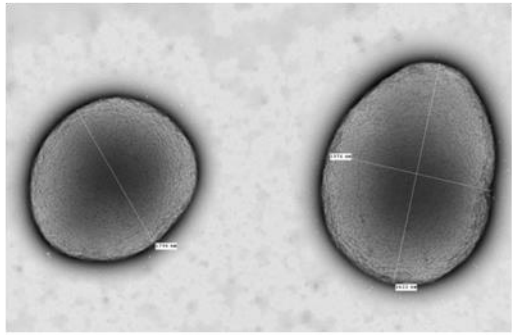
EC-C1 Parent



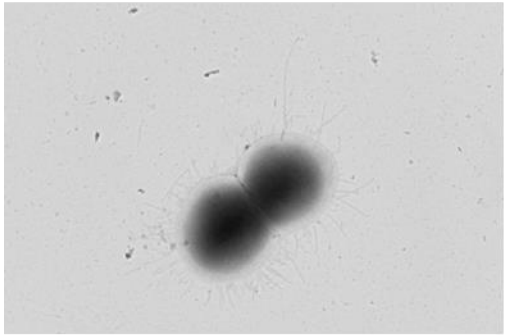
EC-5-a Parent



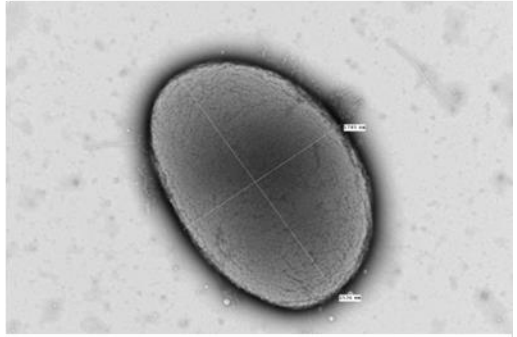
EC-C1 Parent + OP595 (1mg/L)



EC-5 Parent + OP595 (2mg/L)

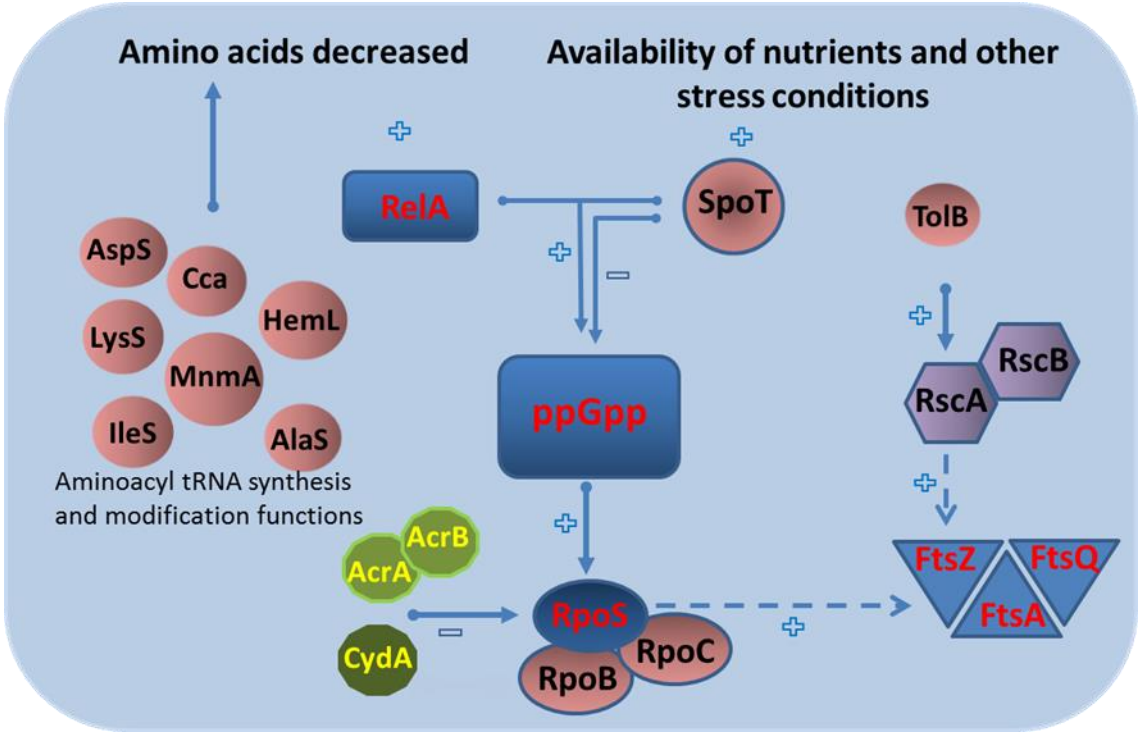


EC-C1 mutant-1 + OP595 (1mg/L)



EC-5-1 mutant-1 + OP595 (2mg/L)

Figure 2. Proposed mechanism(s) of resistance to the antimicrobial activity of OP0595



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