Journal of Global Antimicrobial Resistance 27 (2021) 115–117

Contents lists available at ScienceDirect

Journal of Global Antimicrobial Resistance

journal homepage: www.elsevier.com/locate/jgar

Expression of the *Mycobacterium tuberculosis* RipA cell wall hydrolase in *Streptomyces coelicolor* hampers vancomycin resistance

Editor: Stefania Stefani

Sir,

Antimicrobial resistance (AMR) is a growing health issue today that demands the development of effective new strategies. Combating the growing problem of AMR has proved challenging. There is an active research field investigating compounds that interfere with resistance mechanisms and greatly prolong the use of existing drugs. Therefore, it is fundamental to define the mechanisms of AMR and how they are regulated. Previous work has shown that antibiotic resistance is a dynamic phenomenon that can be influenced by nutrition [1–3], urging us to revisit existing AMR testing protocols. Vancomycin is an antibiotic used in the clinic as a last-resort treatment for many life-threatening bacterial infections, including those caused by methicillin-resistant *Staphylococcus aureus* (MRSA). However, vancomycin resistance has been reported in *S. aureus* and other important *Enterococcus* pathogens, giving rise to dangerous nosocomial infections. In previous works [1–3], it has been evidenced that inorganic phosphate (Pi) induces hypersensitivity to vancomycin in vancomycin-resistant *Streptomyces coelicolor* M145, suggesting that this nutrient may play a key role in vancomycin resistance repression. Bacteria encounter multiple stresses in their natural environments, including nutritional starvation and the action of different cell-wall-stressing agents. Several researchers, including myself, have provided evidence of the role played by different cell-wall-stressing agents in bacterial susceptibility to distinct antibiotics, as reviewed in [4]. For example, it has been noted that the autolytic activity of some glycopeptide-intermediate *S. aureus* strains (GISA) is reduced compared with that of glycopeptide-susceptible strains [4]. Furthermore, it has been reported that lysozyme decreased the vancomycin tolerance of a series of *S. coelicolor* isolates [3].

This work aimed to investigate the effect of the expression of a well-known mycobacterial cell wall hydrolase (RipA) on the vancomycin resistance mechanism of the model organism *S. coelicolor*. Depletion of this enzyme (MSMEG3153) was shown to drastically change the rod-shaped morphology of *Mycobacterium smegmatis* to a filament-branched *Streptomyces*-like morphology [5]. The morphological defect of the mutant could be functionally complemented by the *Mycobacterium tuberculosis ripA* orthologue (rv1477), which is 60% identical to that of *M. smegmatis*, but not by another *ripA*-like paralogue, such as *ripB* (rv1478). Both RipA and RipB are endopeptidases belonging to the NlpC/p60 family and hydrolyse the β-(1,4)-glycosidic linkage between *N*-acetylmuramic acid and *N*-acetylglucosamine. The *ripA* gene is essential for effective cell division in *M. tuberculosis* and *M. smegmatis* [5,6], whereas *ripB* is not [5]. In vitro analyses have shown that purified *M. tuberculosis* RipA protein (Rv1477) is capable of hydrolysing cell wall material isolated from several bacteria, including *Streptomyces* spp. [5]. The *S. coelicolor* SCO2135 gene is the best match of three of the five NlpC/p60-like genes in *M. tuberculosis* (rv1478/*ripB*, rv2190c/*ripC* and rv1566c) and the second best match of the other two (rv1477/*ripA* and rv0024) (see Fig. 1A). An interesting question is whether the filament-branched shape of *Streptomyces* is due to the lack of expression of these types of mycobacterial hydrolases or whether there are other mechanisms of morphology control. To answer this question, and also to check the effect of overexpression of *M. tuberculosis* RipA in the vancomycin resistance pattern of *S. coelicolor*, a series of *S. coelicolor* strains was developed. The effect of constitutive overexpression of SCO2135 and SCO2136 was also studied. SCO2136 is the first gene in a bicistronic operon that codes for an NlpC/p60 family endopeptidase [347 amino acids (aa)]. SCO2135, the downstream gene, encodes a 338-aa protein that also contains an NLPC_P60 domain in its C-terminal region. Therefore, both genes encode predicted endopeptidases similar to NlpC/p60 family hydrolases. This genetic organisation (Fig. 1B) resembles that of *ripA* and *ripB* genes in mycobacterial species [5].

To study the effect of *M. tuberculosis* RipA in *S. coelicolor*, a synthetic version of the gene was constructed and introduced in the bacterium. To construct a synthetic version functional in *S. coelicolor* and ready to be secreted by the TAT secretion system of the bacterium, the gene was engineered as follows. The C-terminal region of the RipA protein (321–472 aa) was fused to the signal peptide (27 aa) of the *S. coelicolor* DagA protein, which is secreted by the TAT system. The synthetic gene (*ripA*-152C) was codon-optimised for a high GC content codon usage (specific for *Streptomyces* spp.) and in vitro synthesized by the company Biomatik. This 152-aa truncated RipA protein contains the sole catalytic domain of the enzyme and lacks the prodomain (40–320 aa) that blocks the catalytic domain of the enzyme in the full-length version. Ruggiero et al. observed by circular dichroism spectroscopy that the prodomain is not needed for enzyme folding and that the release of the prodomain activates RipA activity [7].

The open reading frames of *ripA*-152C, SCO2135 and SCO2136 were transcriptionally coupled to the *S. coelicolor* constitutive and strong *rpsL* promoter, contained in the pRL conjugative and integrative *Streptomyces* vector [3], giving pRL-ripA-152C, pRL-SCO2135 and pRL-SCO2136, respectively (Fig. 1C). Fusion of the *neo* gene (conferring neomycin resistance) with the *rpsL* promoter was previously shown to confer constitutive neomycin resistance to a *S. coelicolor* recombinant carrying the genetic fusion, indicating that the vector works properly [3]. The three constructed vectors (pRL-ripA-152C, pRL-SCO2135 and pRL-SCO2136) were checked by sequencing, introduced into *S. coelicolor* by conjugation and their corresponding phenotypes were studied. For each genetic construction, two recombinant strains were isolated and analysed in the experiment (Fig. 1D). This approach is usually performed when integrative vectors are used in order to discard the association of a

https://doi.org/10.1016/j.jgar.2021.08.013
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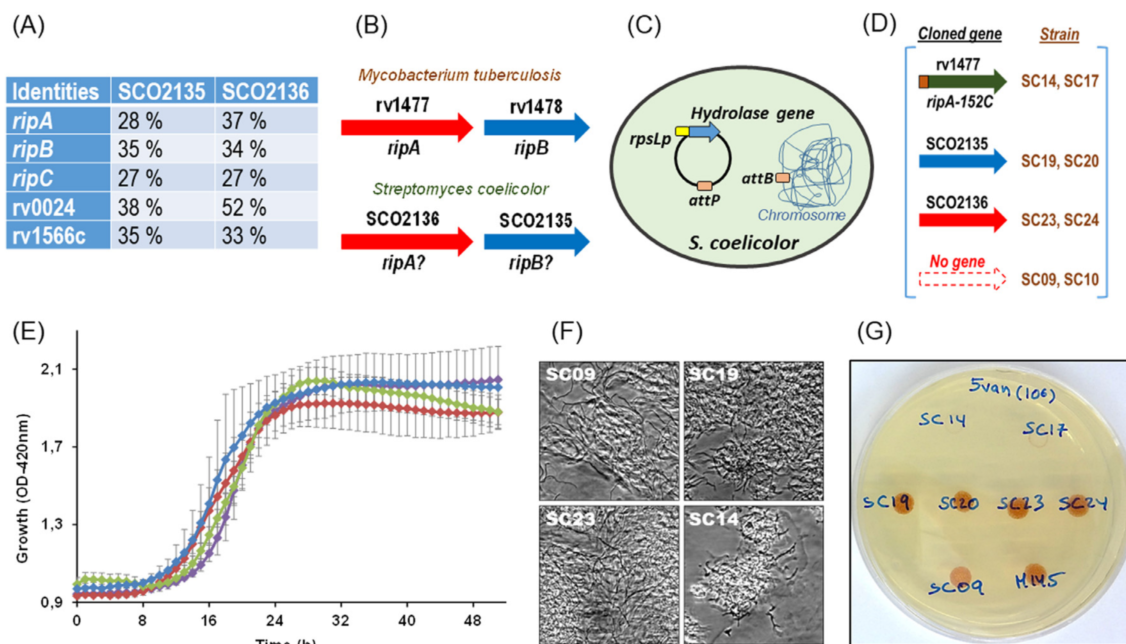

Fig. 1. Effect of constitutive overexpression of genes belonging to the NlpC/p60 family on the growth, morphology and vancomycin resistance of *Streptomyces coelicolor*. (A) Table showing the amino acid identities for each of the NlpC/p60-like proteins in *Mycobacterium tuberculosis* and the *S. coelicolor* SCO2135 and SCO2136 proteins. (B) Genetic organisation of *ripA, ripB*, SCO2135 and SCO2136 genes. (C) Schematic representation of the vectors introduced in *S. coelicolor*. (D) Recombinant strains obtained for each of the gene constructions. (E) Growth of the distinct *S. coelicolor* strains in trypticase soy agar (TSA) with no vancomycin addition. Growth is shown as a mean value of 24 replicates for each genetic construction (12 for each recombinant strain). Purple, pRL; brown, pRL-SCO2135; green, pRL-SCO2136; blue, pRL-ripA-152C. (F) Microscopy images of *S. coelicolor* cells from trypticase soy broth liquid cultures with 1 μg/mL vancomycin. (G) Antimicrobial susceptibility test of the *S. coelicolor* strains developed in this work. In all cases, 10^6 cells were incubated at 30°C for 4 days in TSA with 5 μg/mL vancomycin, which corresponds with the MIC_{50} of vancomycin-resistant *S. coelicolor* strain M145 (no vector) in this medium.

given phenotype with a polar effect produced by an unusual integration of the plasmid in the chromosome, rather than to a real effect of the gene introduction.

Growth was determined by measuring the optical density at 420 nm using a FLUOstar® Optima fluorometer (BMG Labtech) and 96-well microplates. To each well containing 100 μL of solid trypticase soy agar medium, 5 μL of a stock dilution containing 10^6 cells was added. Plates were then incubated at 30°C for 52 h and samples were measured automatically every hour. As shown in Fig. 1E, no effect on growth of the bacterium in comparison with the control strain carrying only the pRL vector was observed in any of the strains expressing the recombinant enzymes. Moreover, the filament morphology of the cells did not change in the absence of vancomycin or with subinhibitory concentrations (i.e. 1 μg/mL) of the antibiotic (Fig. 1F). On the other hand, when a vancomycin concentration (i.e. 5 μg/mL) representing the MIC_{50} value (minimum inhibitory concentration required to inhibit growth of 50% of the bacterial population) was added to the medium, the strain carrying the *ripA*-152C synthetic gene showed a lethal phenotype (see Fig. 1G). Therefore, overexpression of RipA, but not SCO2135 or SCO2136, enhances the antibiotic lethal effect of vancomycin in *S. coelicolor.* In conclusion, overexpression of any of these genes did not cause a decrease in growth or a morphological change in the bacterium when no vancomycin (or a subinhibitory concentration of the antibiotic) was added. Whether SCO2135 or SCO2136 contains a prodomain that block its catalytic domain, as is the case of RipA, or whether any of the other 10 proteins of the NlpC/p60 family, which are predicted to be encoded in the *S. coelicolor* genome, accounts for the RipA function is not yet known.

In summary, in this work the combinatorial effect of two different cell-wall-acting compounds, i.e. a hydrolytic enzyme and a glycopeptide antibiotic, was studied. The work provides scope for further studies of antibiotic resistance control aimed at understanding the influence of different cellular stresses in antimicrobial treatments.

Acknowledgments

The author thanks Prof. Jose Antonio Salas (University of Oviedo, Oviedo, Spain) for assisting him with the coordination of the EU VANRESTREP project and providing work space in his laboratory. The author also thanks Prof. Jeff Errington (Newcastle University, Newcastle upon Tyne, UK) for his support in the construction of the *ripA* synthetic gene.

Funding: This work was supported by the Regional Government of Castilla y León and the EU-FEDER [CLU 2017-09 and UIC 071]. The author was supported at the University of Oviedo by a European H2020-MSCA-IF-2016 Fellowship [UE-18-VANRESTREP-740080].

Competing interests: None declared.

Ethical approval: Not required.

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Revised 8 August 2021