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Functional interplay between the ATP binding cassette Msr(D) protein and the membrane facilitator superfamily Mef(E) transporter for macrolide resistance in *Escherichia coli*

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Abstract

Macrolides have wide clinical applications in the treatment of community-acquired respiratory tract infections, among which streptococci are the most frequent causative agents. An active efflux-based mechanism of macrolide resistance, referred to as the M phenotype in streptococcal isolates, has been associated with the presence of *mef* genes that encode a subset of major facilitator superfamily (MFS) transporters like Mef(E). An *msr*(D) gene, adjacent to and co-transcribed with *mef* in the presence of erythromycin, has also been implicated in drug efflux, but its role remains elusive. Msr(D) belongs to the ATP binding cassette (ABC) proteins and harbors two fused nucleotide-binding domains with no membrane-spanning domains. The present work indicates that the major resistance traits of the M phenotype in *Escherichia coli* may be due to Msr(D) and not to Mef(E). Fluorescence microscopy using Mef(E) tagged with GFP linked low efficacy of the chimera in conferring macrolide resistance with improper subcellular localization. The active role of Msr(D) in directing Mef(E)-GFP to the cell poles was demonstrated, as was synergistic effect in terms of levels of resistance when both proteins were expressed. A trans-dominant negative mutation within ABC Msr(D) affecting MFS Mef(E) strongly suggests that both proteins can interact in vivo, and such a physical interaction was supported in vitro. This is the first reported example of a functional interplay between an ABC component and an MFS transporter. The direct involvement of Msr(D) in the efflux of macrolides remains to be demonstrated.

Keywords: ATP binding cassette; Membrane facilitator; Macrolide resistance

1. Introduction

The worldwide use of macrolide antibiotics in the treatment of respiratory tract infections in both animals and humans has created favorable conditions for selection of resistant bacterial strains, not only among Gram-positive pathogens, the natural macrolide targets, but also among commensals (Liu et al., 2009; Ojo et al., 2004; Woodford and Livermore, 2009). Efflux of macrolide drugs is one of the two most prevalent mechanisms of resistance adopted by streptococci to circumvent the blockade of their ribosomes. This mode of macrolide resistance, which is disseminated by mobile genetic elements (MGEs) among clinical isolates of *Streptococcus pyogenes* and *Streptococcus pneumoniae*, deserves special attention (Goossens, 2009; Varaldo et al., 2009).

Routine laboratory antibiotic susceptibility testing identified the macrolide efflux phenotype in streptococcal isolates as the M phenotype, i.e. resistance to 14- and 15-membered ring macrolides, but not to 16-membered ring macrolides nor to lincosamides (Montanari et al., 2001). Expression of the M phenotype in *S. pneumoniae* is macrolide-inducible through synthesis of a 2.8 kb-long transcript encompassing two contiguous genes, *mef* and *msr*(D), organized into a conserved

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operon structure from within the very diverse MGEs disseminating resistance (Gay and Stephens, 2001; Varaldo et al., 2009). The function of the gene products was determined by single and double deletions combined with cell measures of ^{14}C-erythromycin accumulation, and it was shown that the *mef* and *msr*(D) mutants and double mutants consistently accumulated more erythromycin than their macrolide-resistant parental strains (Ambrose et al., 2005). However, while each protein contributes to an apparent efflux, it remains unclear as to how Msr(D) alone or in association with Mef can function to expel macrolide antibiotics from bacterial cells (Ambrose et al., 2005; Daly et al., 2004).

Mef proteins belong to the major facilitator superfamily (MFS) of transporters (Saier et al., 1999). Three variants of the *mef* gene, *mef*(A), *mef*(E), *mef*(I), are currently recognized among streptococci (Cochetti et al., 2005); however, as the deduced polypeptides share more than 80% amino acid identity, they have been considered a unique resistance determinant, designated Mef(A) by the consensus nomenclature (Roberts et al., 1999). Phylogenetically, the 405-aa variant proteins fall into the DHA3 family which is a diverse and moderately sized cluster whose members exhibit limited sequence similarities with canonical MFS antiporters (Law et al., 2008; Saier et al., 1999). To date, all of the functionally characterized DHA3 proteins are involved in exporting various hydrophobic drugs and siderophores. When expressed in *Escherichia coli*, Mef(A) from *S. pyogenes* was shown to be able to confer resistance to 14-membered ring macrolides such as erythromycin as well as to 15-membered ring macrolides such as azithromycin, but not to 16-membered ring macrolides such as spiramycin (Clancy et al., 1996). Since then, the M phenotype has logically been attributable to the activity of Mef proteins (Ambrose et al., 2005; Daly et al., 2004; Gay and Stephens, 2001; Goossens, 2009; Liu et al., 2009; Montanari et al., 2001; Varaldo et al., 2009).

Msr(D) belongs to the ATP binding cassette (ABC) superfamily of proteins (Bouige et al., 2002). These ATPases are mainly involved in transport processes, but only a few have been implicated in antibiotic export (Bouige et al., 2002; Davidson et al., 2008). The canonical ABC drug exporters comprise two transmembrane domains (TMDs) which usually impart substrate specificity, and two nucleotide-binding domains (NBDs) which couple ATP hydrolysis to drug efflux. Like Vga(A), which was biochemically characterized by us (Jacquet et al., 2008), Msr(D) carries no TMDs and contains two asymmetric NBDs separated by a linker domain of approximately 120 amino acids. These two dual ABC ATPases are representative of the antibiotic resistance (ARE) subfamily in the phylogenetic classification of ABC systems (Bouige et al., 2002; Chesneau et al., 2005). To date, all ARE ATPases await membrane partners in order to be considered as transporters (Kerr et al., 2005). The present work provides several lines of evidence that Msr(D) is a promiscuous ABC protein capable of conferring significant levels of resistance to macrolides in *E. coli* with or without Mef(E), an MFS transporter described as a macrolide efflux pump. The combined action of the ABC and MFS proteins was shown to be truly synergistic, since Msr(D) not only increased macrolide resistance levels conferred by Mef(E), but also broadened its spectrum. Their molecular interaction was supported by genetic and biochemical data.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Ultracompetent cells from *E. coli* TOP10 strain (Invitrogen) were used either for gene cloning or site-directed mutagenesis. *E. coli* AG100A, which is susceptible to macrolides by disruption of the *acrAB* gene cluster (Okusu et al., 1996), was used as a recipient strain for all recombinant plasmids to study the functionality of the gene constructs. Bacteria were routinely grown in Luria–Bertani (LB) medium at 30 °C. Gene expression was controlled by adding isopropyl-β-D-thiogalactopyranoside (IPTG), L-arabinose (Ara) or D-glucose (Glu) at various concentrations. Ampicillin (Ap) and chloramphenicol (Cm) were used at 100 μg/ml and 20 μg/ml, respectively, for plasmid selection. All these chemicals were purchased from Sigma–Aldrich.

2.2. Gene constructs

Amplified genes were cloned and modified according to standard protocols of DNA manipulation (Sambrook and Russell, 2001) using the following kits and reagents: the Zero Blunt Topo PCR cloning kit (Invitrogen), the QIAquick gel extraction and QIAprep spin miniprep kits (Qiagen), the Rapid DNA Ligation kit (Roche), the Quik-Change site-directed mutagenesis kit (Stratagene), *Pfu* DNA polymerase (Stratagene) and various restriction enzymes (Roche). Plasmids used are listed in Table 1. Polypeptide sequences of Msr(D) that we handled (Fig. S1) were deduced from the nucleotide sequences we obtained by double-stranded DNA analysis (Bekman Coulter Genomics). PCR was performed in a PTC-200 thermal cycler apparatus (MJ Research) using the primers, templates and assay conditions summarized in Table S1. Chromosomal DNA from *Streptococcus agalactiae* strain NEMLJ21 was a gift from Prof. Poyart, Groupe Hospitalier Cochin, France. Amplified DNA pieces were first cloned into the pCR4Blunt-TOPO vector and then subcloned to generate each recombinant plasmid of interest: pRBmsr was from pCRVN8, pRBmef from pCRVN9, pVN18 from pCRVN10, pVN20 from pCRVN12, pVN23 from pCRVN14 and pVN26 or pVN27 from pCRVN16. To make pVN17 from pRBmsr, subcloning was done with NcoI and SmaI enzymes. The pVN21 plasmid was obtained by ligating the 1.4 kbp SalI restriction fragment of pVN20 into pVN18. The two pBAD33-derivatives, pVN50 and pVN49, were obtained from pVN18 and pVN32, respectively, by subcloning their 1.6 kbp BamHI–HindIII restriction fragments.

2.3. Antibiotic susceptibility

Overnight LB cultures of AG100A strains harboring the various plasmids used were grown at 30 °C and then appropriately diluted according to the recommendations of the