Constitutive expression of the *ileS-2* gene responsible for high-level mupirocin resistance in *Staphylococcus aureus*

Naira Elane Moreira de Oliveira,^{1†} Elisa D'Avila Costa Cavalcanti,^{2†} Marinella Silva Laport,¹ Maria do Carmo de Freire Bastos¹ and Marcia Giambiagi-deMarval¹

Correspondence

Marcia Giambiagi-deMarval
marciagm@micro.ufrj.br

¹Instituto de Microbiologia Prof. Paulo de Góes, Bloco I, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Cidade Universitária, 21491-590 Rio de Janeiro, RJ, Brazil

²Instituto de Química, Bloco A, Centro de Tecnologia, Universidade Federal do Rio de Janeiro, Cidade Universitária, 21491-590 Rio de Janeiro, RJ, Brazil

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Mupirocin is a topical antimicrobial agent that is used for the treatment of skin and postoperative wound infections, and the prevention of nasal carriage of methicillin-resistant *Staphylococcus aureus* (MRSA). However, the prevalence of mupirocin resistance in *S. aureus*, particularly in MRSA, has increased. High-level mupirocin resistance, with MICs $\geq 512 \mu\text{g ml}^{-1}$, is mediated by the *ileS-2* gene, which is located on conjugative plasmids. In the present study, we investigated whether mupirocin influences the expression of the *ileS-2* gene responsible for high-level mupirocin resistance, and we present some evidence that this gene is not upregulated but constitutively expressed in *S. aureus*.

INTRODUCTION

Staphylococcus are among the major causes of nosocomial infections and nowadays are becoming increasingly difficult to combat because of the emerging resistance to all current antibiotic classes including vancomycin (Fluit *et al.*, 2001). Mupirocin, or pseudomonic acid A, is used to control the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) strains. It is a natural antibiotic, produced by *Pseudomonas fluorescens*, that binds to the isoleucyl-tRNA synthetase (IRS) and arrests protein synthesis by preventing the incorporation of isoleucine into growing polypeptide chains (Yanagisawa *et al.*, 1994). The emergence of high-level mupirocin resistance in staphylococci, associated with a failure to clear the micro-organism in both colonized and infected patients, has been reported (Daskalaki *et al.*, 2009).

The genetics of resistance to mupirocin has been inferred from the MICs shown by the isolates. High-level mupirocin resistance (Mup^R) with MICs $\geq 512 \mu\text{g ml}^{-1}$ has been associated with high-molecular-mass plasmids (30 to 50 kb) harbouring the *ileS-2* gene, which encodes a modified IRS enzyme. Thus, *S. aureus* high-level mupirocin-resistant strains harbour two distinct IRS enzymes: one mupirocin-resistant enzyme encoded by the plasmid *ileS-2* gene and the

usual mupirocin-sensitive enzyme encoded by the chromosomal wild-type *ileS-1* (Gilbart *et al.*, 1993). Alternatively, low- or intermediate-level mupirocin resistance (Mup^I), with MICs ≥ 4 to $\leq 256 \mu\text{g ml}^{-1}$, shows either the presence of a mutated copy of the native *ileS-1* gene on the chromosome (Gilbart *et al.*, 1993) or a copy of the *ileS-2* gene on the chromosome in addition to the native *ileS-1* gene (Ramsey *et al.*, 1996; Fujimura *et al.*, 2001).

Regulation of gene expression generally acts at the level of transcription by either induction or repression. Alternatively, some enzymes are not strongly controlled at all, being expressed constitutively (Watson *et al.*, 2004). In this report, we investigated whether the plasmid *ileS-2* gene associated with high-level mupirocin resistance was constitutively expressed or induced in the presence of mupirocin in *S. aureus*.

METHODS

Bacterial strains. The strains used in this study were *S. aureus* RN8411 (Projan & Archer, 1989), as a Mup^S control, and *S. aureus* JJ1, a transconjugant derivative of strain RN8411 harbouring the plasmid pMG1 responsible for high-level mupirocin resistance (Mup^R) that we characterized previously (Bastos *et al.*, 1999). Both strains were cultivated in brain heart infusion (BHI) broth (Difco) at 37 °C.

Growth in the presence of mupirocin. Strain JJ1 was grown in BHI broth for 18 h at 37 °C. Cells were diluted in BHI broth to OD₆₆₀ 0.05 and separated into two different samples. After 1 h of incubation at 37 °C, 512 $\mu\text{g mupirocin ml}^{-1}$ were added to one of the samples. The

†These authors contributed equally to this work.

Abbreviations: IRS, isoleucyl-tRNA synthetase; MRSA, methicillin-resistant *Staphylococcus aureus*.

cultures were then maintained at 37 °C and the OD₆₆₀ was determined at 1 h intervals.

Metabolic labelling of cells and gel electrophoresis. Overnight cultures were diluted in BHI broth to 10⁷ c.f.u. ml⁻¹ and incubated for 30 min at 37 °C. After centrifugation at 12 000 g for 5 min, cells were resuspended at 10⁸ c.f.u. ml⁻¹ in 100 µl samples of methionine-free minimum essential medium (Gibco) containing 200 µCi ³⁵S-methionine ml⁻¹ (Amersham) (Laport *et al.*, 2001). All aliquots (RN8411, JJ1 or JJ1 supplied with 512 µg mupirocin ml⁻¹) were maintained at 37 °C for 30 min. The labelling period was ended by placing the suspensions in an ice bath and cells were collected by centrifugation at 12 000 g for 5 min. After two washes in 0.85 % NaCl, the cells were incubated in the presence of 0.2 mg lysostaphin ml⁻¹ (Sigma), for 2 h at 37 °C. After that, an equal volume of 0.5 M Tris/HCl (pH 7.2) buffer containing 4 % SDS, 10 % β-mercaptoethanol, 20 % glycerol and 0.1 % bromophenol blue was added. The cell suspensions were boiled for 5 min and cellular debris was sedimented by centrifugation at 12 000 g for 5 min and discarded. Aliquots containing equal radioactive counts from each set of samples were subjected to SDS-PAGE (7.5 %). The gels were stained with Coomassie blue, destained, dried and exposed to X-ray films.

RNA isolation and hybridization. Total RNA from exponentially growing *S. aureus* JJ1 or RN8411 was isolated essentially using the acid-guanidine-thiocyanate-phenol/chloroform extraction method described by Chomczynski & Sacchi (1987). To analyse the synthesis of *ileS-2* mRNA, JJ1 cells were supplied with 512 µg mupirocin ml⁻¹ and incubated for 10 min. Subsequently, the cells were disrupted by incubation with 0.2 mg lysostaphin ml⁻¹, in TE buffer for 2 h at 37 °C. RNA (5 µg per sample) was analysed by Northern blotting on a 1.2 % agarose-formaldehyde denaturing gel and blotted to a nitrocellulose membrane as described elsewhere (Sambrook & Russell, 2001). The RNA blot was probed with a fragment of the *S. aureus* JJ1 *ileS-2* gene PCR amplified according to Nunes *et al.* (1999) and labelled with [α -³²P]dCTP. To confirm the integrity of the RNA and the loading of equal amounts of RNA in the Northern blot gel, a replica gel was stained with ethidium bromide (0.5 µg ml⁻¹).

RESULTS AND DISCUSSION

Growth of Mup^R *S. aureus* in the presence of mupirocin

To analyse the influence of mupirocin on the rate of growth of mupirocin-resistance cells, the Mup^R transconjugant JJ1 was incubated at 37 °C either in the absence or in the presence of mupirocin (512 µg ml⁻¹) and growth was monitored at 1 h intervals. In the presence of mupirocin, the cultures showed slower growth (Fig. 1). When overnight cultures were grown and kept, throughout the experiment, in the presence of mupirocin (Fig. 1), the same slower growth was observed indicating that this delay may result from the inhibition of the sensitive mupirocin IRS, encoded by the chromosomal *ileS-1* gene in the tested strain. All cultures were in the exponential growth phase after 4 h of incubation.

Expression of the *ileS-2* gene

In order to analyse the expression of the *ileS-2* gene at the translational level, in the presence of mupirocin, RN8344

Fig. 1. Influence of mupirocin on the growth of the Mup^R *S. aureus* JJ1 strain. Cells were incubated at 37 °C without mupirocin (■) or with the addition of 512 µg mupirocin ml⁻¹ after 1 h (indicated by an arrow) (▲). As a control, another culture was pre-incubated in the presence of 512 µg mupirocin ml⁻¹ and always kept in the presence of the drug (▼). At 1 h intervals, the OD₆₆₀ was determined for each condition.

Mup^S and its derivative transconjugant JJ1 Mup^R were submitted to isotopic labelling in the presence of [³⁵S]methionine. In Fig. 2(a), we observed the presence of a high-molecular mass protein with a molecular mass (120 kDa) compatible with that of the IRS, which contains 1024 amino acids. This protein was present in the transconjugant JJ1 (lane ii) but not in the strain RN8411 (lane i). The presence of mupirocin in the JJ1 culture (lane iii) did not change the pattern of *IleS-2* protein expression.

To analyse transcription of the *ileS-2* gene, an equal amount of total RNA isolated from JJ1 cells, incubated either in the absence or in the presence of 512 µg mupirocin ml⁻¹ for 10 min was analysed by Northern blotting using an *ileS-2* specific probe. Three different transcripts of approximately 9.5, 5.3 and 3.4 kb were detected (Fig. 2b). The 9.5 kb mRNA may correspond to a polycistronic transcript containing three copies of the *ileS-2* gene, which seems to be arranged in tandem on the Mup^R plasmids (Morton *et al.*, 1995). The mRNAs of 5.3 and 3.4 kb may correspond to either degradation or single monocistronic transcripts since the *ileS-2* gene is 3075 bp in size (GenBank accession number X75439.1). No significant difference was detected in the amount of *ileS-2* transcripts in the mupirocin-treated cells compared to the untreated ones, reinforcing the idea of constitutive expression of the *ileS-2* gene.

We have previously shown that high-level mupirocin resistance was correlated with the presence of a 35 kb relaxable plasmid in a MRSA strain isolated in the hospital of Universidade Federal do Rio de Janeiro, Brazil (Bastos *et al.*, 1999). This plasmid, named pMG1, was transferred to strain RN8411 during overnight filter-matings and one of the transconjugants was named JJ1. In this work, the transconjugant JJ1 Mup^R was shown to constitutively

Fig. 2. Influence of mupirocin on protein synthesis and mRNA transcription of the *ileS-2* gene in *S. aureus* JJ1. (a) *S. aureus* cells supplied with mupirocin or not supplied with mupirocin were incubated in the presence of ^{35}S [methionine] (200 mCi ml^{-1}) for 30 min at 37°C . The labelled proteins were separated by SDS-PAGE (7.5%) and visualized by autoradiography. The lanes correspond to the following strains: *S. aureus* RN8411 Mup^S (i), *S. aureus* JJ1 Mup^R labelled in the absence (ii) or in the presence (iii) of $512 \mu\text{g mupirocin ml}^{-1}$. The arrow indicates the position of the *IleS-2* protein of 120 kDa absent in lane i. (b) Northern blot of total RNA ($5 \mu\text{g}$ per lane) of *S. aureus* JJ1 incubated for 10 min at 37°C without (–), or with (+) $512 \mu\text{g mupirocin ml}^{-1}$, separated on a 1.2% gel, transferred to a nylon membrane and hybridized to an *ileS-2*-specific probe.

express the *ileS-2* gene responsible for mupirocin resistance. When the growth of Mup^R JJ1 strain was analysed, the presence of mupirocin seemed to slow down the growth of the resistant strain when compared with the growth of this strain in the absence of the drug. As stated above, this may be due to the inhibition of the mupirocin-sensitive IRS encoded by the chromosomal *ileS-1* gene (Gilbart *et al.*, 1993) since the same delay was observed when cells were pre-incubated and maintained throughout the experiment in the presence of mupirocin. Additionally, in the presence of mupirocin, neither transcription nor translation of the *ileS-2* gene was changed resulting in no alteration in expression of mRNA and protein, respectively. Taken together, these results suggest that the expression of the high-level mupirocin-resistant *ileS-2* gene is not upregulated in the presence of mupirocin.

To the best of our knowledge, this is the first report that reveals the constitutive expression of the *ileS-2* gene, i.e. its expression is independent of the presence of the antimicrobial mupirocin. These data broaden our understanding of the genetics of high-level mupirocin resistance in *S. aureus*.

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