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Active antimicrobial efflux in *Staphylococcus epidermidis*: building up of resistance to fluoroquinolones and biocides in a major opportunistic pathogen

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Objectives: To analyse the efflux-mediated response of *Staphylococcus epidermidis* to ethidium bromide (EtBr), a substrate of multidrug efflux pumps (EPs).

Methods: The susceptible reference strain *S. epidermidis* ATCC 12228 was exposed to a step-wise adaptation to EtBr. The resulting EtBr-adapted strains were characterized regarding their antibiotic and biocide susceptibility by MIC determination and evaluation of efflux activity by re-determination of MICs in the presence of known efflux inhibitors and real-time fluorometry. Mutations in the QRDR of *grlA* and *gyrA* were screened by sequencing. The expression levels of *S. epidermidis* homologues of the main *Staphylococcus aureus* EP genes were quantified by RT–qPCR.

Results: Exposure to EtBr led to a gradual increase in resistance to antimicrobials, with the final EtBr-adapted strain, ATCC 12228_EtBr, displaying phenotypic resistance to fluoroquinolones and reduced susceptibility to several antiseptics and disinfectants, although no mutations were detected in the QRDR of the *grlA/gyrA* genes. A reduction in the MICs of fluoroquinolones and selected biocides promoted by efflux inhibitors suggested an efflux-mediated response to EtBr exposure. Detailed analysis of the EtBr-adapted strains detected a gradual increase in efflux activity. Gene expression assays revealed a temporal activation of *S. epidermidis* EPs, with an early response involving *norA*, SE2010 and SE1103 followed by a late response mediated by *norA*, which coincided with the occurrence of the mutation $^{-1}$A→T in the *norA* promoter region.

Conclusions: This study demonstrated that *S. epidermidis* has the potential to develop a multiple resistance phenotype mediated by efflux when exposed to a non-antibiotic substrate of multidrug EPs.

Introduction

Despite its commensal nature, *Staphylococcus epidermidis* is an important nosocomial pathogen responsible for life-threatening infections associated with the use of medical devices and in immunocompromised individuals, whose management is hindered by frequent resistance to antimicrobials.[1,2] However, in opposition to *Staphylococcus aureus*, our understanding of *S. epidermidis* antimicrobial resistance mechanisms, particularly efflux driven, is very limited. To date, almost 10 chromosomally encoded multidrug efflux pumps (EPs) have been characterized in *S. aureus* with 20 additional putative multidrug EPs.[3–5] In *S. epidermidis*, only homologues of the *S. aureus* EPs NorA/B/C have been predicted so far.[6] Several studies from our group and others have shown that, in *S. aureus*, efflux contributes to resistance to fluoroquinolones and other antibiotics as well as antiseptics and disinfectants, in addition to promoting the emergence of MDR phenotypes.[7–12]

A strategy to assess the role of native EPs is to characterize isogenic strains that differ in the expression of these pumps.[13] In the present study, we detail the adaptation of an *S. epidermidis* susceptible strain to ethidium bromide (EtBr), a non-antibiotic substrate of several multidrug EPs. The resulting EtBr-adapted cultures showed increased efflux activity, correlated with higher levels of resistance to antimicrobials and EP genes overexpression, which ultimately led to phenotypic resistance to fluoroquinolones and reduced susceptibility to biocides.

Materials and methods

EtBr-adaptation process

The susceptible reference strain *S. epidermidis* ATCC 12228 was subjected to a step-wise EtBr-adaptation process. The strain was grown at 37 °C in tryptone soya broth (TSB, Oxoid, UK) supplemented with doubling increasing

concentrations of EtBr, starting from 0.25 mg/L (half the MIC of EtBr) up to 32 mg/L. After 23 days of growth and eight passages (Figure S1, available as Supplementary data at *JAC* Online), a strain adapted to 32 mg/L EtBr was obtained and denominated ATCC 12228_EtBr.

Antimicrobial susceptibility testing

MICs of antibiotics and biocides (Sigma–Aldrich, USA) were determined, in triplicate, by the two-fold broth microdilution method and evaluated according to EUCAST recommendations.[14]

MIC determination in the presence of efflux inhibitors (EIs)

MICs were re-determined in the presence of thioridazine, chlorpromazine, reserpine and verapamil as described above, except for the addition of each EI at half or lower its MIC prior to the inoculum.[12]

Real-time fluorometry

EtBr efflux assays were performed following incubation of bacteria with 400 mg/L verapamil (half MIC) and the most suitable EtBr concentration to maximize intracellular EtBr loading (Figure 1).[12,15] Efflux activity was characterized by the slope (m) and the relative index of efflux activity (RIE).[12]

Macrorestriction analysis

SmaI (New England Biolabs, USA) restriction fragments of chromosomal DNA were resolved by PFGE.[16]

Screening of mutations conferring fluoroquinolone resistance

The QRDR of *grlA* and *gyrA* and the promoter region of *norA* were amplified, sequenced (primers in Table S1) and analysed with MEGA v 6.0.

In silico search for S. epidermidis putative multidrug EPs

Homologues of *S. aureus* native multidrug EPs[5] and additional putative EPs were identified using public databases and *in silico* freeware (see footnote to Table S3).

Gene expression analysis

Total RNAs were isolated by the Trizol method.[12] RT–qPCR experiments were carried out with equivalent RNA quantities and the QuantiTect SYBR Green RT–PCR Kit (QIAGEN, Germany) (primers in Table S1). Relative gene expression of isogenic strains was assessed by the comparative threshold cycle (C_T) method,[17] comparing each mRNA with the parental ATCC 12228 strain. Genes *gyrB* and 16S rDNA were used as reference controls; negative and genomic DNA contamination controls were included.

Results and discussion

Our understanding of the contribution of native multidrug EPs to antimicrobial resistance in *S. epidermidis* is still limited. To explore the capacity of *S. epidermidis* to produce an efflux-mediated response towards toxic stimuli, we exposed the susceptible strain ATCC 12228 to EtBr in a step-wise manner, an approach previously applied to *S. aureus*.[13] The occurrence of contaminations or major chromosomal rearrangements during this process were ruled out by SmaI-PFGE patterns, which remained unaltered (data not shown). MIC values of EtBr, fluoroquinolones and biocides increased gradually following the increase in EtBr concentration, with an onset at exposure to the EtBr MIC (0.5 mg/L, passage P2). Phenotypic resistance to ciprofloxacin (MIC >1 mg/L) was detected following exposure to 4 mg/L EtBr (passage P5) (Table 1). Strain ATCC 12228_EtBr displayed an EtBr MIC of 32 mg/L, 64-fold higher than the MIC for the parental ATCC 12228 strain. It also presented MIC increases of 2-fold to 16-fold to several biocides including cetrimide, benzalkonium chloride and cetylpyridinium chloride (Table 1). Most importantly, ATCC 12228_EtBr showed a final ciprofloxacin MIC of 4 mg/L, 16-fold higher than the MIC for the parental ATCC 12228 strain. An even higher increment (32-fold) was observed for the norfloxacin MIC. Mild MIC increases were also observed for aminoglycosides and for erythromycin (Table 1). The emergence of fluoroquinolone resistance was not related to mutations in the QRDR of *grlA/gyrA* genes, which remained unaltered.

To ascertain the mechanism underlying the resistance phenotype, we evaluated the efflux activity throughout the EtBr-adaptation process. MICs for ATCC 12228_EtBr of EtBr, fluoroquinolones and selected biocides were re-determined in the presence of EIs, considering an MIC reduction equal or higher than 4-fold as indicative of efflux inhibition (Table S2).[10,12] All EIs were able to reduce, although in different degrees, the MICs of the antimicrobials tested. Particularly, they could revert the phenotype of ciprofloxacin resistance. These results suggest that the ATCC 12228 parental strain exhibits basal efflux activity and the increase in MICs observed was due to an augmented efflux activity. Fluorometric assays confirmed this hypothesis, evidencing that the efflux activity increased gradually (lower slope values, higher RIE values), following the increase in EtBr concentration, with isogenic strain ATCC 12228_EtBr displaying the highest efflux activity ($m = -0.447$, $RIE = 0.810$) (Figure 1a).

To establish which putative multidrug EP(s) were involved in this augmented efflux, we searched for homologues of *S. aureus* multidrug EPs and other putative multidrug transporters. Twenty-five putative transporters were identified in the genome of *S. epidermidis* ATCC 12228 strain and other fully sequenced strains, which included the already predicted homologues of NorA/B/C,[6] as well as other putative members of all the major secondary transporter families (Table S3). Five genes were selected for expression assays; the homologues of *norA* (SE0466), *norB/norC* (SE0196 and SE2010), the gene SE1103, encoding a transporter from the Multidrug and Toxic Compound Extrusion (MATE) family (the transporter family of MepA) and SE0458, a homologue of the *S. aureus* regulator gene *mgrA*. Figure 1(b) presents the expression levels of these genes throughout the EtBr-adaptation process. Increasing expression levels were detected for all genes tested, except SE0196, up to passage P6 (8 mg/L EtBr), whereas in ATCC 12228_EtBr (P8, 32 mg/L EtBr), only the *norA* homologue was overexpressed. Sequencing of the *norA* promoter region revealed a $^{-1}$A→T transversion in ATCC 12228_EtBr (Figure 1c), which occurred only at this last passage. These results suggest that the resistance phenotype observed in ATCC 12228_EtBr was mainly mediated by NorA efflux activity in a similar manner to the one observed following the EtBr adaptation of a susceptible *S. aureus* strain.[13] Yet, the temporal response of *S. epidermidis* to the EtBr challenge revealed an early response with cells increasing the levels of expression of *norA*, SE2010 and SE1103 as the EtBr pressure rises. Increasing the EtBr selective pressure, a distinct late response emerged, with a mutation occurring in the *norA* promoter of ATCC 12228_EtBr and an overexpression of *norA* while the remaining EP genes resumed their initial basal expression levels (Figure 1b).

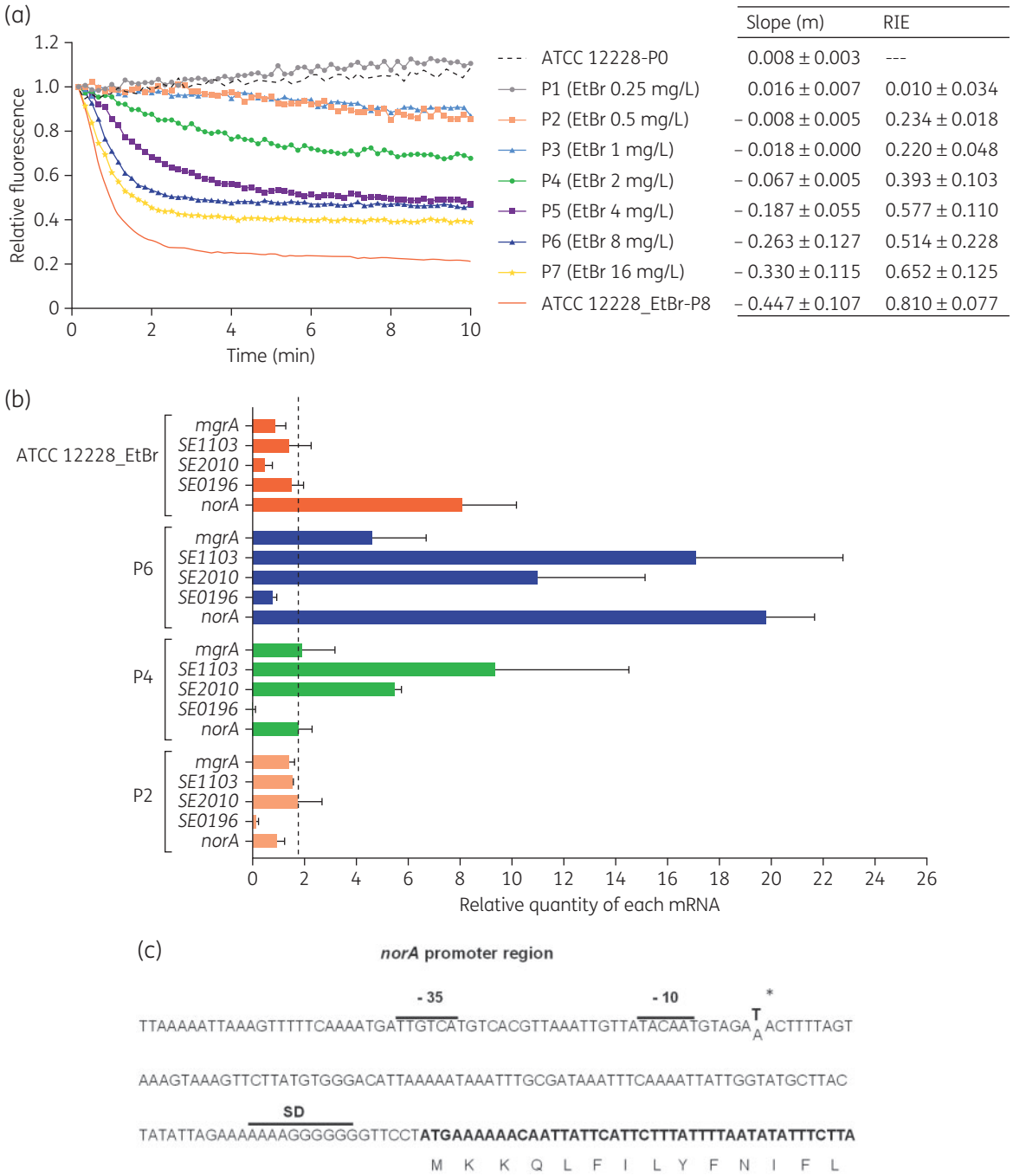

Figure 1. Assessment of efflux activity by real-time fluorometric EtBr efflux assays (a), gene expression levels in EtBr-adapted strains of four putative *S. epidermidis* multidrug EPs and an *mgrA* homologue (b) and analysis of the *norA* promoter region (c). (a) Real-time fluorometric assays were conducted in the presence of 0.4% glucose. EtBr-loaded cells were obtained by incubation with 400 mg/L verapamil plus the following EtBr concentrations: 0.125 mg/L (ATCC 12228-P0 to P4); 0.25 mg/L (P5 and P6); and 0.5 mg/L (P7 and ATCC 12228_EtBr-P8). The data presented were normalized against the data obtained under conditions of no efflux (cells incubated without glucose in the presence of 400 mg/L verapamil). The slope (m) of the EtBr efflux curves was calculated by a linear regression of the values obtained in the first 2 min of the assay and it relates to the rate of EtBr efflux under each condition tested.[12] The RIE values were calculated as described previously and allow the direct comparison of the EtBr efflux activity of each culture at each step of the exposure (P1 to P8) relative to ATCC 12228 prior to EtBr exposure (P0).[12] (b) Gene expression levels for passages P2, P4, P6 and P8 (ATCC 12228_EtBr) were quantified by the comparative method in relation to strain ATCC 12228. The results are presented as the mean and standard deviation of at least three independent assays performed with total RNA from independent extractions. Overexpression was considered for values superior to 2 (cut-off value represented by the dashed line). (c) Nucleotide sequence of the *norA* promoter region of ATCC 12228, depicting the −35 and −10 consensus sequences, the putative site of transcription initiation (*) and the Shine–Dalgarno (SD) sequence. At position −1, strain ATCC 12228 presents an adenine, whereas strain ATCC 12228_EtBr displays a thymine (in bold).

Table 1. MICs of EtBr, antibiotics and biocides for the isogenic strains obtained during each passage of the EtBr adaptation of the parental *S. epidermidis* ATCC 12228 strain

	MIC (mg/L) for each isogenic strain								
Antimicrobial	P0	P1	P2	P3	P4	P5	P6	P7	P8
EtBr	0.5	0.5	1	2	4	4	8	16	32
Antibiotics									
ciprofloxacin	0.25	0.25	0.5	0.5	1	**2**	**2**	**2**	**4**
norfloxacin	0.5	0.5	2	2	4	4	8	16	16
oxacillin	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
penicillin	128	128	128	128	128	64	64	64	64
ampicillin	16	16	16	16	16	16	16	16	8
vancomycin	2	2	2	2	2	2	2	2	2
gentamicin	0.03	0.03	0.03	0.03	0.06	0.06	0.06	0.06	0.125
kanamycin	0.5	0.5	0.5	0.5	0.5	1	1	1	1
erythromycin	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1
tetracycline	16	16	16	16	16	16	16	16	16
chloramphenicol	4	4	4	4	4	4	4	4	4
Biocides									
cetrimide	2	2	4	4	4	4	8	8	8
benzalkonium chloride	1	1	2	2	2	2	2	2	4
chlorhexidine digluconate	1	1	1	1	1	1	2	2	2
tetraphenylphosphonium bromide	32	32	128	128	128	256	256	256	512
dequalinium chloride	1	1	2	2	2	2	2	4	4
cetylpyridinium chloride	0.25	0.25	1	1	1	1	2	2	4

P0, parental *S. epidermidis* ATCC 12228; P1, ATCC 12228 grown in TSB + 0.25 mg/L EtBr; P2, ATCC 12228 grown in TSB + 0.5 mg/L EtBr; P3, ATCC 12228 grown in TSB + 1 mg/L EtBr; P4, ATCC 12228 grown in TSB + 2 mg/L EtBr; P5, ATCC 12228 grown in TSB + 4 mg/L EtBr; P6, ATCC 12228 grown in TSB + 8 mg/L EtBr; P7, ATCC 12228 grown in TSB + 16 mg/L EtBr; P8, *S. epidermidis* ATCC 12228_EtBr, grown in TSB + 32 mg/L EtBr.
Values in bold highlight ciprofloxacin MIC values categorized as phenotypic resistance as evaluated by the EUCAST recommendations.[14]

A similar response has been observed in *S. aureus* exposed to constant concentrations of EtBr, ciprofloxacin or cetrimide; a first response involving the overexpression of several EP genes, followed by an overall decrease in expression levels and the overexpression of specific EP genes.[12]

In this work, we further characterized the expression analysis of secondary multidrug EPs other than *norA*, in one of the few reports associating *S. epidermidis* EPs to antimicrobial resistance. Previous studies have associated NorA to gatifloxacin resistance in an endophthalmitis isolate[6] and to EtBr resistance.[18] The latter recent study also linked mutations in the *norA* promoter or 5′ untranslated regions with gene overexpression.[18] The $^{-1}$A→T mutation we encountered adds to the already described mutations in the *S. epidermidis norA* promoter that could affect *norA* expression levels by altering the efficiency of *norA* transcription.

In summary, our study reveals that similarly to *S. aureus*,[12] *S. epidermidis* has the potential to develop a resistance phenotype mediated solely by efflux following exposure to a non-antibiotic substrate of multidrug EPs. Our results support the pivotal role of NorA on the response to antimicrobial challenge. Yet, they also add a temporal perspective on the activation of different EP genes by *S. epidermidis*; an early response with activation of several EP genes followed by a late response mediated by *norA*. Together, these studies show that both *S. aureus* and *S. epidermidis* respond to antimicrobial challenge by overexpressing available EPs, until a mutation occurs that stabilizes the expression of a specific EP or alters the compound target. The ability of *S. epidermidis* for such an efflux-mediated response highlights the need of a deeper understanding of the role of native EPs in the development of antimicrobial resistance by this pathogen.

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Transparency declarations

None to declare.

Supplementary data

Figure S1 and Tables S1 to S3 are available as Supplementary data at *JAC* Online.

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