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NorC, a New Efflux Pump Regulated by MgrA of *Staphylococcus aureus*

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NorC, a new efflux pump, like NorB, contributes to quinolone resistance that includes resistance to moxifloxacin and sparfloxacin in *Staphylococcus aureus*. *norC* expression, like that of *norB* and *tet38*, is negatively regulated by MgrA, and overexpression of both *norC* and *norB* contributes to the quinolone resistance phenotype of an *mgrA* mutant.

NorA, NorB, and Tet38 are previously described efflux transporters of the major facilitator superfamily in *Staphylococcus aureus* that are under the control of MgrA, a global regulator that also affects diverse virulence factors (5, 7, 11, 12). MgrA acts as a negative regulator of NorB, and NorB overexpression in an *mgrA* mutant contributes to low-level quinolone resistance but does not fully account for the quinolone resistance phenotype of the *mgrA* mutant. We report here the identification of an additional chromosomally encoded multiple-drug resistance efflux pump, termed NorC, which is also negatively regulated by *mgrA* and which in addition to NorB contributes to quinolone resistance in an *mgrA* mutant.

S. aureus cells (Table 1) were grown in brain heart infusion (BHI) broth, and *Escherichia coli* cells were grown in Luria-Bertani (LB) broth. *norC* and *cat* genes were amplified by PCR, using primers containing BamHI and EcoRI for *norC* and PvuII for *cat*. The conditions were as follows: 1 cycle for 3 min at 94°C; 30 cycles for 45 s at 94°C, 1 min at 48°C, and 1 min at 72°C; and 1 cycle for 10 min at 72°C. *norC* was cloned into pGEM3-zf(+) and then subcloned into pSK950 to generate pQT11. pGEM3-zf(+)-*norC* was digested with MscI and ligated to the PvuII-digested *cat* gene, generating construct pGEM3-zf(+)-*norC*::*cat*. *norC*::*cat* was subcloned into pCL52.2, generating pQT12. Allelic exchange was carried out as previously described to generate mutant QT9 (*norC*::*cat*) (12). To create a *norC mgrA* double mutant, we transferred *norC*::*cat* from QT9 into QT1 using bacteriophage φ85 as described previously (11).

MICs were determined on BHI agar supplemented with serial twofold drug dilutions. Transformants containing pSK950, pQT8, and pQT11 were plated on BHI with 5 μg/ml tetracycline and incubated at 30°C.

Primers amplifying a 400-bp amplicon of SA0098 (5′-GTA GAAACGAATGTCGGACCAC-3′ and 5′-AATGGCATC ATTGGCCATA-3′) and a 200-bp amplicon of *norC* (5′-AAA TGGTTCTAAGCGACCAA-3′ and 5′-ATAAATACCTGA AGCAACGCCAAC-3′) were synthesized. Reverse transcriptase PCR (RT-PCR) used SA0098 as standard RNA and *norC* as target RNA (4). SA0098 was cloned into pSK950 and then introduced into mutant QT9 (*norC*::*cat*). RNA from a transformant (0.01 to 0.20 μg) was added to the RT-PCR mix. ISP794 and QT1 RNA amounts were 0.15 μg for each reaction. Conditions were as follows: 1 cycle for 30 min at 45°C; 1 cycle for 2 min at 94°C; 28 cycles for 45 s at 94°C, 45 s at 48°C, and 30 s at 72°C; and 1 cycle at 10 min for 72°C. Photographs of ethidium bromide-stained gels were scanned and analyzed using the NIH Scion Image program (version 6.1), as described previously (2).

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristic(s)	Reference(s) or source
Strains		
S. aureus		
RN4220	8325-4 r^-	6
ISP794	8325-4 *pig*-131	10
QT1	ISP794 *mgrA*::*cat*	12
QT5	ISP794 *norB*::*cat*	11
QT6	ISP794 *mgrA*::*cat norB*::*cat*	11
QT9	ISP794 *norC*::*cat*	This study
QT10	ISP794 *mgrA*::*cat norC*::*cat*	This study
E. coli		
DH5α	F^- φ80d*lacZΔM15* Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 phoA hsdR17*(r_K^- m_K^-) *supE44*λ *thi-1 gyrA96 relA1*	Gibco-BRL
Plasmids		
pGEM3-zf(+)	2.9-kb *E. coli* cloning vector, Ap^r	Promega
pWN2018	10.5-kb *S. aureus* transcriptional promoter-probe vector Cm^r	1, 2, 13
pBF8-30	315 bp containing the entire promoter of *norA* cloned upstream of the *blaZ* gene of pWN2018	1
pLI50	Shuttle cloning vector (Ap^r Cm^r)	9
pCL52.2	Temperature-sensitive *E. coli-S. aureus* shuttle vector	9
pSK950	10.5-kb plasmid carrying the *attP* site of phage L54a, replicon of pE194, Tc^R, Em^R (*S. aureus*)	8
pQT8	pSK950-*norB*	11
pQT11	pSK950-*norC*	This study
pQT12	pCL52.2-*norC*::*cat*	This study
pWN2018-P_{norC}	200 bp containing the entire putative promoter of *norC* cloned upstream of the *blaZ* gene of pWN2018	This study

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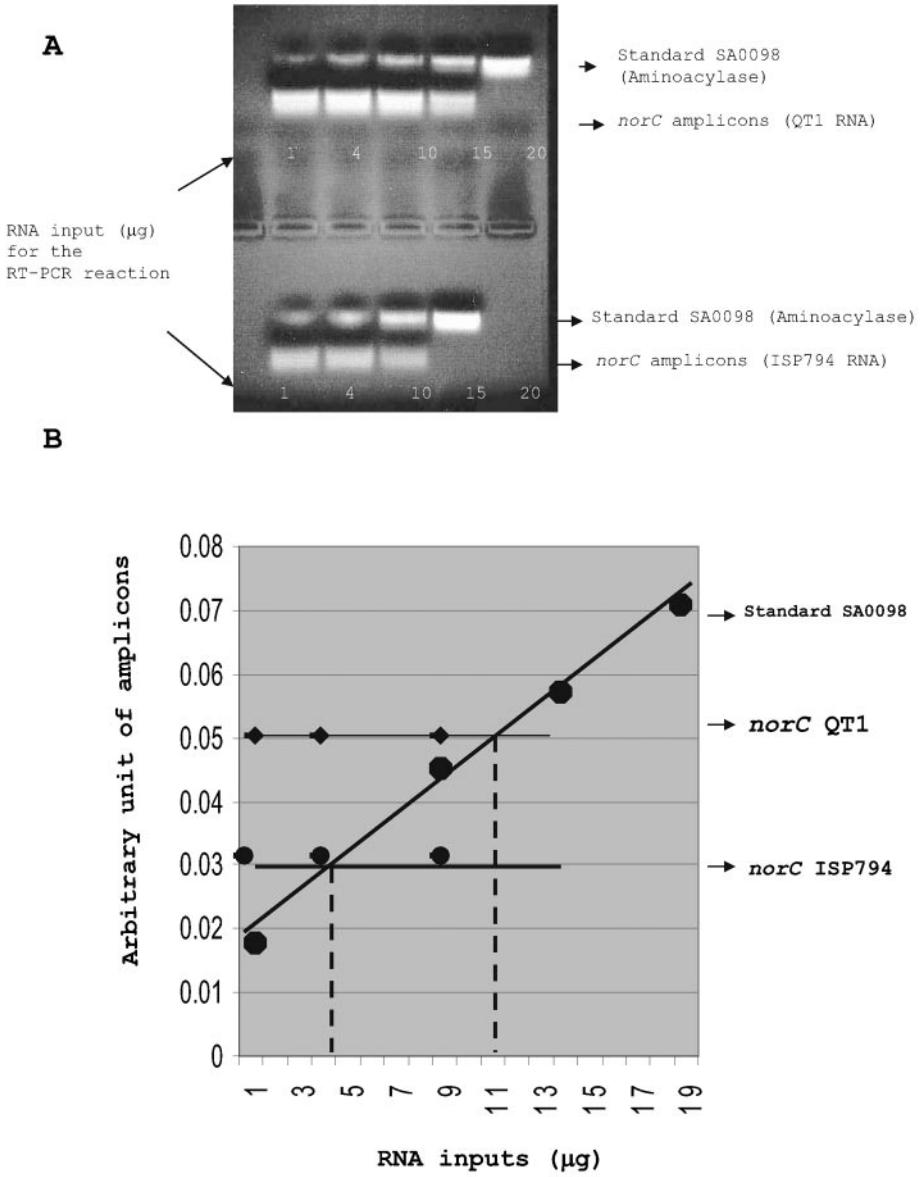

FIG. 1. (A) Noncompetitive quantitative RT-PCR using ORF SA0098 RNA as standard RNA and *norC* mRNA as target RNA. The output signals of the two amplification products were plotted versus the amount of standard RNA. (B) Graphic representation of the quantitative RT-PCR. The target signal (*norC* amplicon) remains constant, and the standard curve (SA0098 amplicon) is linear. The intersection point between the two lines is the equivalence point and indicates the arbitrary value for the *norC* amplicons.

Primers for the *norC* promoter (5′-GCAGCTGTGGTACCAGATGGTGA-3′ and 5′-ACTGCAGTTTCATTCATGTTAGTTA-3′) containing KpnI and PstI were synthesized (restriction sites underlined). Conditions were as follows: 1 cycle for 3 min at 94°C; 30 cycles for 45 s at 94°C, 45 s at 48°C, and 20 s at 72°C; and 1 cycle for 10 min at 72°C. The product was digested with KpnI and PstI and cloned into pWN2018, generating pWN2018-P_{norC}. Cells containing pWN2018-P_{norC} were grown in Trypticase soy broth at 37°C to an optical density at 600 nm of 0.9. The assays used nitrocefin as the substrate as described previously (1, 2). The activities were expressed in micromoles of nitrocefin hydrolyzed per hour per gram of cell protein.

Because overexpression of *norB* in *mgrA* mutant QT1 only partially explained the quinolone resistance phenotype of QT1 (11), we analyzed further microarray data comparing QT1 and its parent strain ISP794 and identified another putative transporter gene, annotated as open reading frame (ORF) SA0099 in the *S. aureus* N315 genome. This ORF showed a 2.9-fold increase in mRNA in QT1. ORF SA0099 is predicted to encode a protein identical to *S. aureus* SbtA, which is listed in GenBank (S. Sinjee and L. J. V. Piddock). The predicted protein, which we have named NorC, had 61% amino acid identity with NorB. To confirm the increased expression of *norC*, we performed noncompetitive RT-PCR under conditions for which no competition occurred between the target and the standard, and the output signals of the amplification

TABLE 2. Activity of quinolones against *norC* and *mgrA* strains and other mutant strains

Strain (plasmid)[a]	Reserp[b]	MIC (μg/ml) of[c]:						
		NOR	CIP	SPAR	MOXI	GEMI	GARE	PREMA
ISP794	−	0.5	0.25	0.125	0.06	0.015	0.03	0.06
	+	0.5	0.25	0.125	0.06	0.015	0.03	0.06
QT1	−	4	2	0.5	0.25	0.06	0.25	0.25
	+	1	0.5	0.125	0.06	0.015	0.03	0.06
QT5	−	0.5	0.25	0.125	0.06	0.008	0.03	0.06
	+	0.5	0.25	0.125	0.06	0.008	0.03	0.06
QT6	−	1	0.5	0.25	0.125	0.015	0.06	0.125
	+	0.5	0.25	0.125	0.06	0.015	0.03	0.06
QT9	−	0.5	0.25	0.06	0.03	0.015	0.03	0.06
	+	0.5	0.25	0.06	0.03	0.015	0.03	0.06
QT10	−	1	0.5	0.25	0.125	0.03	0.06	0.125
	+	0.5	0.25	0.25	0.06	0.015	0.03	0.06
ISP794(pSK950)	−	0.5	0.25	0.125	0.06	0.015	0.03	0.06
	+	0.5	0.25	0.125	0.06	0.015	0.03	0.06
ISP794(pQT8)	−	4	0.5	0.25	0.25	0.06	0.125	0.125
	+	1	0.25	0.125	0.06	0.015	0.03	0.06
ISP794(pQT11)	−	2	0.5	0.25	0.25	0.015	0.125	0.125
	+	1	0.5	0.125	0.06	0.015	0.03	0.06
QT9(pQT11)	−	2	0.5	0.25	0.125			
	+	0.5	0.5	0.125	0.06			
QT10(pQT11)	−	2	1	0.25	0.25			
	+	1	0.25	0.125	0.06			

[a] Strains harboring plasmids pSK950, PQT8, and pQT11 were grown in the presence of tetracycline (5 μg/ml).
[b] −, reserpine absent; +, reserpine present.
[c] NOR, norfloxacin; CIP, ciprofloxacin; SPAR, sparfloxacin; MOXI, moxifloxacin; GEMI, gemifloxacin; GARE, garenoxacin; PREMA, premafloxacin.

products were plotted versus the amount of RNA. *norC* RNA levels in QT1 were increased 2.75-fold relative to those in ISP794 (Fig. 1). Northern hybridization also showed a similar increase (data not shown).

Upstream of *norC* is ORF SA0098 (1,179 bp), which is predicted to encode a putative aminoacylase/carboxypeptidase. Using the Neural Network Promoter Prediction program (http://www.fruitfly.org/seq_tools/promoter.html), we found a putative *norC* promoter, P_{norC}, which overlapped the end of SA0098. To assess the role of this promoter in the expression of *norC*, we constructed pWN2018-P_{norC} (*norC* promoter-*blaZ* fusion) and introduced it into ISP794 and QT1 *mgrA*. We then compared the β-lactamase expression from this construct with that from pBF8-30, a *norA* promoter-*blaZ* fusion (1), measuring the initial linear rate of nitrocefin color development from 0 to 5 min. In the parent strain ISP794, there was little difference in β-lactamase expression levels for *norC* and *norA* promoter fusions. In QT1, in contrast, a 3.7-fold increase in expression was observed for the *norC* promoter, with little change in the expression level for the *norA* promoter relative to expression in ISP794. Thus, P_{norC} has promoter activity that increases in an *mgrA* mutant background. Although incubation of a 200-bp DNA fragment upstream of *norC* containing P_{norC} with crude cell extracts of ISP794 and QT1 exhibited differing patterns of DNA mobility shift, no mobility shift was seen with 2 μg purified histidine-tagged MgrA protein, which causes a mobility shift of *norA* promoter DNA (data not shown) (3, 11, 12). Thus, heterologously expressed MgrA appears not to interact directly with P_{norC}, suggesting that other cellular factors or modified MgrA is the direct regulator of *norC* expression.

To assess the effect of *norC* overexpression on resistance to quinolones, plasmid pQT11 with cloned *norC* and control plasmid pSK950 were introduced separately into ISP794. Relative to ISP794(pSK950), ISP794(pQT11) showed fourfold increases in MICs of norfloxacin, garenoxacin, and moxifloxacin; twofold increases in MICs of ciprofloxacin, sparfloxacin, and premafloxacin; and no change in the MIC of gemifloxacin. Increases in MICs were inhibited by reserpine (Table 2).

In *norC* knockout mutant QT9 derived from ISP794, there was no change in the MICs of most quinolones, except for a twofold decrease in MICs of sparfloxacin and moxifloxacin relative to ISP794. In contrast, the *norC mgrA* double mutant QT10 exhibited increased susceptibility to all quinolones tested relative to the *mgrA* mutant and to within twofold of that of ISP794. Transformants QT9(pQT11) and QT10(pQT11) showed increases in quinolone resistance to levels the same as those for ISP794(pQT11) and QT1, respectively (Table 2).

Thus, *norC* contributes to the resistance phenotype of an *mgrA* mutant.

NorC represents a third multiple-drug resistance efflux pump, in addition to NorA and NorB, that can cause low-level quinolone resistance when overexpressed. Expression of *norC*, also like that of *norA* and *norB*, is regulated by *mgrA*. MgrA appears to function as a negative regulator of *norC*, as it does for *norB* and *tet38*, which encodes tetracycline resistance (11). Overexpression of *mgrA* from a plasmid, in contrast, acts positively on expression of *norA* in the ISP794 genetic background (12). Thus, MgrA plays a central role in modulating expression of at least four genes encoding efflux pumps and in modulating resistance to quinolones and tetracycline (11, 12).

The phenotypes of NorC-overexpressing strains and mutant QT9 (*norC*::*cat*) establish a role for NorC in low-level reduced susceptibility to sparfloxacin and moxifloxacin in *S. aureus*. NorB overexpression also causes low-level resistance to sparfloxacin and moxifloxacin, but NorB is apparently not expressed in the wild-type strain to a level sufficient to affect susceptibility to these agents, since the susceptibility of mutant QT5 (*norB*::*cat*) did not differ from that of its wild-type parent (11).

The resistance profiles of the two double mutants, QT6 (*mgrA norB*) and QT10 (*mgrA norC*), further suggest that NorB and NorC efflux pumps act in concert to generate the quinolone resistance phenotype when MgrA is inactivated. Thus, MgrA acts to coordinately regulate the expression of at least four efflux pumps in *S. aureus*. Although it appears to act directly on the *norA* promoter (12), the effects of MgrA on other promoters, including P_{norC}, appear to be indirect (11), indicating that regulatory elements in addition to MgrA are important for controlling expression of several efflux pumps in *S. aureus*.

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