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Analyses of Multidrug Efflux Pump-Like Proteins Encoded on the *Staphylococcus aureus* Chromosome

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Drug resistance is a therapeutic challenge in *Staphylococcus aureus* infections, and efflux is an important component of it. The activity of efflux proteins (pumps) can reduce susceptibility to important antimicrobial agents, predispose the bacterium to target-based mutations, enhance environmental survival via biocide tolerance, and increase nosocomial infection risk (1, 2).

S. aureus possesses a large complement of genes encoding putative drug and toxin efflux pumps, the majority of which reside on the chromosome and remain uncharacterized. The annotations of three genomes (*S. aureus* strains 8325, COL, and N315) were examined, and all genes encoding putative secondary active drug efflux proteins, which are dependent on ion gradients for substrate translocation, were identified (http://www.ncbi.nlm.nih.gov/genome/154). The *S. aureus* 8325 genome also was submitted

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TABLE 1 Open reading frames on the *S. aureus* chromosome encoding putative efflux proteins

Open reading frame in strain:							
NCTC 8325	COL	N315	Gene[a]	Protein length (amino acids)	TMS[b]	Predicted protein family and substrate(s)[c]	Reference(s)
0042	0070	NP[d]		406	12	MFS, macrolides	
0058	0086	0099	*norC*	462	14	MFS, multidrug	12
0062	0090	0103		290	10	DMT, drugs/amino acids	
0078	0103	0115		418	12	MFS, multidrug	
0099	0122	0132	*tet38*	450	14	MFS, tetracycline	11
0143	0163	0172		416	12	MFS, multidrug	
0246	0261	0263		458	14	MFS, quinolones	
0315	0405	0323	*mepA*	451	12	MATE, multidrug	9
0556	0620	0531		466	12	MFS, proline/betaine	
0681	0733	0628		406	12	MFS, sugars	
0703	0754	0650	*norA*	388	12	MFS, multidrug	13, 14
0740	0790	0684		244	8	DMT, drugs/amino acids	
0952	1021	0874		402	12	MFS, glycolipids	
1448	1475	1269	*norB*	463	14	MFS, multidrug	11
1876	1809	1580		393	12	MFS, multidrug	
2418	2157	1970	*lmrS*	480	14	MFS, lincomycin/multidrug	15
2419	2158	1971	*sepA*	157	4	Novel,[e] multidrug	16
2420	2159	1972	*sdrM*	447	14	MFS, multidrug	17
2435	2170	1982		397	12	MFS, macrolides	
2525	2252	2056		1,055	12	RND, multidrug, AcrB-like	
2531	2257	2061		403	12	MFS, drugs	
2629	2347	2142		643	15	MFS, lincomycin/multidrug	
2700	2413	2203	*mdeA*	452	14	MFS, multidrug	8
2725	2437	2222		403	12	MFS, bicyclomycin/teicoplanin	
2740	2449	2233		466	14	MFS, quinolones/multidrug, NorB-like	
2752	2460	2241		375	12	MFS, chloramphenicol/multidrug	
2762	2471	2250	*norD*	397	12	MFS, drugs	18
2797	2504	2283		397	12	MFS, unknown	
2818	2523	NP		387	12	MFS, multidrug	
2843	2548	2322		311	10	DMT, drugs/amino acids	
2866	2566	2339		822	12	RND, multidrug, MmpL-like[f]	

[a] If previously characterized.
[b] TMS, transmembrane segments. Secondary structure predictions of putative pump proteins were made by the transmembrane helix prediction algorithm TMHMM at www.cbs.dtu.dk/services/TMHMM.
[c] Predicted by TransAAP and PSI-BLAST tools available at www.ncbi.nlm.nih.gov. MFS, major facilitator superfamily; MATE, multidrug and toxic compound extrusion; DMT, drug/metabolite transport; RND, resistance-nodulation-division.
[d] NP, not present within the genome.
[e] Structurally dissimilar to known pump families.
[f] MmpL, Mycobacterial membrane protein large, involved in mycolic acid and lipid transport (19).

to the Transporter Automatic Annotation Pipeline (TransAAP) to identify putative transport protein-coding genes (www.membranetransport.org/transaap/TransAAP_login.html). Data from these manual and automated analyses were combined to identify 31 genes of interest (Table 1). Ten of the 31 proteins have been established as drug efflux pumps and were not investigated further.

PCR was used to amplify the remaining 21 genes from the chromosome of *S. aureus* SH1000, incorporating KpnI and SacI sites at the 5′ and 3′ ends, respectively (3). The PCR products were cloned into pALC2073 digested with these enzymes, placing coding regions under the control of the tetracycline-inducible *xyl/tetO* promoter (4). Plasmids were amplified in *E. coli*, electroporated into *S. aureus* RN4220, and then transferred into the *norA*-disrupted background strain *S. aureus* SA-K2124 (SH1000 *norA*::*lacZ*) by transduction (5, 6). Control strains included SA-K2703 and SA-K3756, which are SA-K2124 derivatives harboring pALC2073 and pALC2073-*norA*, respectively.

Test strains were grown in cation-supplemented Mueller-Hinton broth (SMHB) including chloramphenicol (10 μg/ml) for maintenance of pALC2073-based constructs. The MICs of biocides, dyes, and antimicrobial agents were determined in duplicate for all strains by broth microdilution according to CLSI guidelines (7). The microtiter plates included tetracycline (0.05 μg/ml) to induce the expression of cloned genes. Data were normalized to those for SA-K2703, and MIC increases of 4-fold or greater were considered significant.

Compared to the MICs for strain SA-K2703, the expression of *norA* in SA-K3756 resulted in 4-fold to 32-fold increases in MICs to numerous substrates (indicated by underlining below). However, no significant MIC increases for acriflavine, benzalkonium chloride, berberine, cetrimide, chlorhexidine, crystal violet, 4,6-diamidino-2-phenylindole (DAPI), dequalinium, ethidium bromide, fusidic acid, gentamicin, Hoechst 33342, lincomycin, linezolid, norfloxacin, pentamidine, pyronin Y, sodium dodecyl sulfate, teicoplanin, tetracycline, tetraphenylphosphonium bromide, and rhodamine 6G were detected upon induction of the expression of any of the 21 putative drug efflux genes. Transcriptional and translational efficiency may have contributed to our results, but based on the phenotype conferred by the expression of *norA* from pALC2073 in SA-K3756, this appears unlikely.

The vast majority of previously characterized *S. aureus* multidrug resistance efflux pump genes were identified using transcriptional profiling or shotgun cloning approaches while investigating drug-resistant mutants (8–11). The *in silico* approach we used did not rely on a preexisting phenotype. Our goal was to fill a knowledge gap regarding the identity of all efflux proteins encoded within the *S. aureus* genome. It remains feasible that one or more of the genes we examined encodes a protein having a unique substrate profile. It also is possible that other drug transport protein-encoding genes exist in the *S. aureus* genome that were not identified by our approach. Nevertheless, we can conclude that none of the genes evaluated herein encode a protein capable of transporting typical pump substrates.

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