Mutations in the *fabI* promoter associate to other mechanisms of triclosan resistance in *Staphylococcus aureus*

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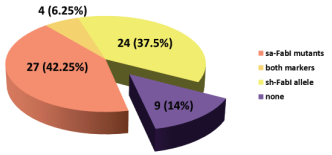
Biohypo Consortium
This study was financed by FP7 EC project KBBE-227258 (BIOHYPO)

BACKGROUND: The biocide triclosan is a chlorinated bis-phenol with a broad spectrum antimicrobial activity. Triclosan, unlike other biocides, at low concentrations has a single intracellular target identified so far: the enoyl-acyl carrier protein (ACP) reductase enzyme (FabI), involved in bacterial fatty acid biosynthesis. From a collection of 1600 *S. aureus* isolates, we have previously shown that 64 strains demonstrated an increased MBC towards triclosan. Mutations in *fabI* gene locus and heterodiploidy for *fabI* have been shown to confer resistance in *S. aureus*. Still, reduced susceptibility in 14% of these strains could not be attributed to one of these mechanisms.



AIM: The present study investigates the molecular nature of *fabI* overexpression in *S. aureus* isolates and in *in vitro* selected mutants with reduced susceptibility to triclosan.

METHODS: Promoter analysis was performed on 38 triclosan resistant clinical strains and 23 laboratory mutants selected after multipassage exposure to triclosan. The promoter region was amplified by PCR and sequenced. Furthermore, 7 laboratory mutants and their 3 corresponding parental strains were subjected to all genome sequencing. The gene expression profile of 4 clinical strains and 4 laboratory mutants with mutations in the promoter regions was compared to their wild type, triclosan susceptible isogenic or prototypical strains. For this purpose we used a self-designed custom array containing probes targeting genes extracted from 2 different *S.aureus* genomes and a variety of genes of plasmid origin.

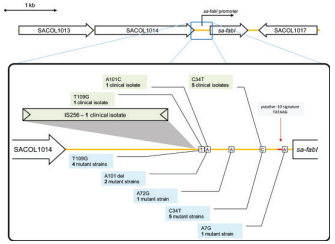
RESULTS

RN4220 derived						ATCC6538 derivd							
MO035		MO034		MO036		d2		d7		MO052			
POS*	MUT	POS	MUT	POS	MUT	POS	MUT	POS	MUT	POS	MUT	Annotation	Comment
										208900	G-T	Pyruvate formate-lyase1 activating enzyme, putative	Val-phe
								226813	G-TA			Globin domain protein	Premature truncation
290822	T-A											Promoter of Conserved hypothetical protein: SAOUHSC_00271	Promoter of SAOUHSC_00271
292879	C-A											Protein of unknown function, DUF600	Ser-Arg
292992	T-C											Conserved hypothetical protein: SAOUHSC_00275	Met-Thr
										919922	T-G	fabI promoter region (T109G)	
		919959A-G										fabI promoter region (A72G)	
919997	C-T											fabI promoter region (C34T)	
				920024A-G								fabI promoter region (A7G)	
920098	G-C											fabI G68C mutation	Gly23Ala
		920331G-T		920331G-T								fabI G301T mutation	Asp101Tyr
								920469	T-C			fabI T439C mutation	Tyr147His
						920641T-G				920641	T-G	fabI T611G mutation	Phe204Cys
										1231020	T-A	mutL. Involved in DNA mismatches repair.	Ile-Asn
								1588812	G-A			Conserved hypothetical protein: SAOUHSC_01679	Ile-Thr
2461195	C-A											Respiratory nitrate reductase, gamma subunit	Cys-Phe
2723733	T-G											Clumping factor B, putative	Glu-Asp

The number of mutations detected by all genome sequencing in the selected laboratory mutants varied from one to nine mutations per genome. Only mutations in the fabI locus (in red) were shared by all the strains and none of the other mutations was common to more than one strain.

Strain	sa-fabI promoter**	sa-fabI	sh-fabI	MIC*	MBC*	Comment
MO036	A7G	mutated	-	4	8	
MO035	C34T	mutated	-	8	8	
MO047	C34T	mutated	-	4	8	
MO049	C34T	mutated	-	4	8	
MO076	C34T	mutated	-	4	8	
CR002	C34T	mutated	-	4	8	
MO034	A72G	mutated	-	8	8	
MO077	A101-	mutated	-	4	32	1 bp deletion
d7	A101-	mutated	-	2	8	1 bp deletion
MO051	T109G	mutated	-	4	8	
MO052	T109G	mutated	-	8	16	
MO053	T109G	mutated	-	4	8	
MO055	T109G	mutated	-	4	8	
QBR-102278-1097	C34T	mutated	-	0.25	32	
QBR-102278-1889	C34T	wt	-	0.5	16	
QBR-102278-1969	C34T	wt	-	0.25	32	
QBR-102278-2095	C34T	wt	-	0.25	32	
QBR-102278-2546	C34T	mutated	+	1	64	
QBR-102278-2363	A101C	wt	+	16	32	
QBR-102278-1052	T109G	wt	+	0.5	64	IS256 insertion

Mutations in the *fabI* promoter were found in 18.4 % of triclosan resistant clinical isolates and half of the laboratory mutants, regardless the molecular mechanism conferring resistance. This suggests that mutations in the *fabI* promoter do not confer resistance by themselves, but are associated to other mechanisms of resistance.



Some of the mutations identified in the clinical isolates were detected also in the series of laboratory mutants, indicating that in this case *in vitro* model mimics selective pressure in the field.

Comparison	fabI promotor mutation	Upregulated probes	Downregulated probes	Fold increase in FabI expression
MO034 vs RN4220	A72G	207	159	4.96
MO035 vs RN4220	C34T	10	-	3.92
MO036 vs RN4220	A7G	2	-	-
MO052 vs ATCC6538	T109G	4	24	3.46
QBR102278-1889 vs ATCC25923	C34T	200	25	4.86
QBR102278-1969 vs ATCC25923	C34T	190	45	6.44
QBR102278-2363 vs ATCC25923	A101C	193	34	4.26
QBR102278-1052 vs ATCC25923	T109G	348	9	47.6

The comparisons between in vitro selected mutants and their isogenic wild type strains identified *fabI* as one of the few significantly up-regulated genes. A similar comparison between triclosan resistant clinical isolates containing mutations in the *fabI* promoter and sensitive prototypical strains also demonstrated a significant up-regulation of the *fabI* gene. In all 4 clinical isolates, 37 genes were commonly up-regulated and 6 down-regulated (see following table). Furthermore, one clinical strain showed a particularly high level of *fabI* gene expression and associated triclosan resistance. This strain was characterized by the insertion of an IS256 element in the promoter region of *fabI*.

Protein product	Gene locus TW20	Gene locus MU50	Fold change
Up-regulated genes			
DNA gyrase subunit A	SATW20_00060 / *gyrA*	SAV0006 / *gyrA*	2.65
Putative flavohemoprotein	SATW20_02420	SAV0240	17.18
Uncharacterized protein	SATW20_04160	SAV0348	2.49
Xanthine phosphoribosyltransferase	SATW20_04540 / *xprT*	SAV0388 / *xprT*	9.9
Putative autolysin (N-acetylmuramoyl-L-alanine amidase)	SATW20_05330	SAV0465	8.04
Uncharacterized protein	SATW20_07380	SAV0663	8.31
Fructose 1-phosphate kinase	SATW20_07740	SAV0699 / *fruB*	3.41
PTS transport system, fructose-specific IIABCcomponent	SATW20_07750 / *fruA*	SAV0700 / *fruA*	8.11
Ribonucleoside-diphosphate reductase	SATW20_08060 / *rir1*	SAV0731 / *nrdE*	2.75
Lipoteichoic acid biosynthesis protein /poly	SATW20_09350 / *dltD*	SAV0935 /*dltD*	4.13
Thioesterase superfamily protein	SATW20_09440	SAV0944	4.29
Enoyl-acyl-carrier-protein reductase (NADH)	SATW20_10080 / *fabI*	SAV:011 / *fabI*	15.81
Sodium:alanine symporter family protein	SATW20_13570	SAV:356 / *alsT*	2.88
Phosphatidylglycerol lysyltransferase	SATW20_13600 / *mprF*	SAV:360 / *fmtC*	5.44
UPF0365 protein	SATW20_15690	SAV:573	3.83
Putative D-serine/D-alanine/glycine transporter	SATW20_16860 / *cycA*	SAV:696 / *aapA*	4.23
Signal transduction protein TRAP	SATW20_18290 / *trap*	SAV:835 / *traP*	14.65
Uncharacterized protein	SATW20_18470	SAV:853	2.8
Nitric oxide synthase oxygenase	SATW20_19090	SAV:914	4.94
Uncharacterized protein		SAV1947	16.6
Phage protein	SATW20_19330 / *hlb*	SAV1954	5.32
Phage protein	SATW20_19350		10.12
Staphylococcal complement inhibitor SCIN	SATW20_19360 / *scn*	SAV1942 / *scn*	65.23
Uncharacterized protein	SATW20_20150	SAV2032	3.11
UDP-N-acetylglucosamine1-carboxyvinyltransferase	SATW20_22380 / *murA1*	SAV2099	8.59
Transcription termination factor Rho	SATW20_22590 / *rho*	SAV2121 / *rho*	3.78
Uncharacterized protein	SATW20_23220	SAV2184	5.52
Xanthine/uracil permeases family protein	SATW20_23870	SAV2253	3.62
Lysostaphin resistance protein A	SATW20_24670	SAV2335	2.78
Putative lipoprotein	SATW20_25000	SAV2368	8.9
Uncharacterized protein	SATW20_25130	SAV2383	4.44
Putative nitrite transporter	SATW20_25330	SAV2403	3.72
Uncharacterized protein	SATW20_25340	SAV2404	5.28
ABC transporter ATP-binding protein	SATW20_25420	SAV2412	15.01
ABC transporter permease	SATW20_25430	SAV2413	7.39
ABC transporter solute-binding lipoprotein	SATW20_25440	SAV2414	3.15
Immunodominant staphylococcal antigen B	SATW20_27760 / *isaB*	SAV2638 / *isaB*	3.28
Down-regulated genes			
Uncharacterized protein	-	SAV0801	0.15
Putative phage infection protein	SATW20_27810	SAV2643	0.37
Fructose-bisphosphate aldolase class I	SATW20_27450 / *fda*	SAV2606	0.03
Putative hydratase	SATW20_01020	-	0.23
Putative N-acetyltransferase	SATW20_28340	-	0.06
Putative transmembrane protein SmpB	SATW20_26360	SAV2515	0.36

CONCLUSION: **We have shown that, C34T, T109G and A101C mutations in the *fabI* promoter region confer *fabI* up-regulation both in clinical isolates and/or laboratory mutants. The insertion of IS256 may further enhance promoter activity. This is the first report on genetic evidence linking promoter mutations and up-regulated expression of the fabI gene.**

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