Supplementary Information for

Real-time imaging of the bacillithiol redox potential in the human pathogen *Staphylococcus aureus* using a genetically encoded bacilliredoxin-fused redox biosensor

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Running title: Bacilliredoxin-fused redox biosensor in *Staphylococus aureus*

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Supplemental Figure legends

Figure S1. Multiple protein sequence alignments of the YphP/YqiW bacilliredoxin homologs from *B. subtilis*, *S. aureus* COL and USA300 and *Staphylococcus epidermidis*. The bacilliredoxins YphP (BrxA) and YqiW (BrxB) of the UPF0403 family from *B. subtilis* were aligned with SACOL1464 and SACOL1558 from *S. aureus* COL, SAUSA300_1321 and SAUSA300_1463 from *S. aureus* USA300 and with SERP1006 and SERP1075 from *S. epidermidis*. The multiple sequence alignment was performed using ClustalW2 and presented using Jalview. Intensity of the blue color gradient is based on 50% identity. The conserved Cys residues are marked with asterisk (*) and highlighted in red.

Figure S2. The Brx-roGFP2 biosensor is not responsive to pH changes *in vitro*. Recombinant Brx-roGFP2 was diluted into 0.1 M potassium phosphate buffer with pH values ranging from 5.8-7.9. The 405/488 nm fluorescence excitation ratio of fully reduced and oxidized Brx-roGFP2 was calculated at different pH values, emission was measured at 510nm (n=4, P=0.0637 Brx-roGFP2 pH 5.8 versus 7.8). Error bars represent standard deviations from the mean. Data are representative of 4 replicates and *P*-values were calculated using a Students unpaired two-tailed t-Test by the graph prism software.

Figure S3. Western blot analysis of Brx-roGFP2 expression in S. aureus COL during the growth in LB medium. *S. aureus* COL Brx-roGFP2 was grown in LB medium and crude protein extracts were analyzed at different times along the growth for Brx-roGFP2 expression using GFP-specific Western blot analysis. Brx-roGFP2 expression increases during the stationary phase in *S. aureus* COL. The protein size marker indicates that the Brx-roGFP2 protein migrates at the correct molecular weight of 46 kDa in *S. aureus* COL *in vivo*.

Figure S4. Fluorescence excitation spectrum of Brx-roGFP2 expressed in *S. aureus* COL (A) and USA300 (B) at different growth phases (OD1, 3, 5) and the effect of the level of Brx-roGFP2 in COL and USA300 on OxD at OD=4 (C, D).

(A, B) Fluorescence intensity and Brx-roGFP2 expression is much lower in USA300 during the log phase at OD1-3 compared to COL (*n*=2; P=0.0001 at OD 2.5).

(C,D) Comparison of serial dilutions of COL and USA300 cells harvested at OD=4 (undiluted, 1:2-fold and 1:3-fold dilutions) showed that the OxD at OD=4 remains constant (C) and is not affected by the different Brx-roGFP2 expression level due to the dilutions. The Brx-roGFP2 level in the different dilutions was analyzed using roGFP2-specific Western blot analysis (D). Mean values are shown and error bars represent the *s.e.m* and *P*-values were calculated using a Students unpaired two-tailed t-Test by the graph prism software.

Figure S5. Comparison of the Brx-roGFP2 response in *S. aureus* COL and the natural *bshC* mutant strain RN4220 during the growth. The Brx-roGFP2 biosensor is constitutively oxidized in the *S. aureus* RN4220 strain due to the natural *bshC* mutation of the NCTC8325-4 lineage (*n*=4; P<0.0001 in all samples). The oxidation degree was calculated based on 405/488 nm excitation ratios with emission at 510 nm and related to the fully oxidized and reduced controls as described in the Methods section. Mean values are shown and error bars represent the *s.e.m* and *P*-values were calculated using a Students unpaired two-tailed t-Test by the graph prism software.

Figure S6. Quantification of Brx-roGFP2 amounts in *S. aureus* COL wild type and the *bshA* mutant during the growth in LB-medium. (A) The protein samples were harvested from the *S. aureus* COL Brx-roGFP2 wild type and the *bshA* mutant at different time point along the growth curves. **(B)** Brx-roGFP2 expression was analyzed using Western blot analyses. **(C)** Purified His-tagged Brx-roGFP2 protein was used standard for calibration. **(D)** Quantification of Brx-roGFP2 in the *S. aureus* strains revealed similar expression levels in the wild type and the *bshA* mutant (*n*=2; P=0.9346 for Brx-roGFP2 amounts in all samples of

WT/*bshA*). Mean values are shown and error bars represent the *s.e.m* and *P*-values were calculated using a Students unpaired two-tailed t-Test by the graph prism software.

Figure S7. BSH-specific non-reducing Western blot analysis of *S. aureus* COL, COL with empty vector pRB473 and COL Brx-roGFP2. *S. aureus* COL, COL-pRB473 and COL Brx-roGFP2 strains were grown in LB medium to an OD_{540} of 2.0, washed and transferred to Belitsky minimal medium and exposed to 100 µM NaOCl for 30 min. The proteins were extracted and subjected to non-reducing SDS-PAGE and BSH-specific Western blot analysis. The *S*-bacillithiolated proteins are increased especially in the 40 kDa range in all strains by NaOCl stress indicating that the Brx-roGFP2 probe does not affect the pattern of *S*-bacillithiolation.

Figure S8. Responses of purified roGFP2 and Brx-roGFP2 proteins *in vitro* to H_2O_2 and NaOCl. Purified roGFP2 and Brx-roGFP2 proteins were exposed to 100 µM-10 mM H_2O_2 **(A, B)** and 20-100 µM NaOCl *in vitro* **(C, D).** The oxidation degree was calculated based on 405/488 nm excitation ratios with emission at 510 nm and related to the fully oxidized and reduced controls. Mean values are shown and error bars represent the *s.e.m* and *P*-values were calculated using a Students unpaired two-tailed t-Test by the graph prism software.

Figure S1


YphP 1 MSMAYEEYMRQLVVPMRRELTGAGFEELTTAEEVENFMEKA--EGTTLVVVNSVCGCAAGLARPAATQAVLQNDKTPDNTVTVF 82
YqiW 1 MNMDFNLFMNDIVRQARQEITAAGYTELKTAEEVDEALTK---KGTTLVMVNSVCGCAGGIARPAAYHS-VHYDKRPDQLVTVF 80
SACOL1464 1 -MNAYDAYMKEIAQQMRGELTQNGFTSLETSEAVSEYMNQVNADDTTFVVINSTCGCAAGLARPAAVAVATQNEHRPTNTVTVF 83
SACOL1558 1 MDMNFDLYMNGVVEQARNEIESAGYEQLTTAEDVDKVLKQ---DGTTLVMINSVCGCAGGIARPAASHA-LHYDVLPDRLVTVF 80
SAUSA300_1321 1 -MNAYDAYMKEIAQQMRGELTQNGFTSLETSEAVSEYMNQVNADDTTFVVINSTCGCAAGLARPAAVAVATQNEHRPTNTVTVF 83
SAUSA300_1463 1 MDMNFDLYMNGVVEQARNEIESAGYEQLTTAEDVDKVLKQ---DGTTLVMINSVCGCAGGIARPAASHA-LHYDVLPDRLVTVF 80
SERP1006 1 -MNGYEAYMKELAQQMRAELTDNGFTSLETSDDVNQYMQNIDNDDTTFVVINSTCGCAAGLARPAAVAVAEQNEVKPDHKVTVF 83
SERP1075 1 MDLNFDLYMNDVVEQARNEIEHAGYHQLTSAEDVDQVLQQ---KGTSLVMVNSVCGCAGGIARPAAAHA-LHYDKLPQRLVTVF 80
* *
YphP 83 AGQDKEATAKMREYFTGQEPSSPSMALLKGKEVVHFIPRHEIEGHDMEEIMKNLTAAFDAHC--- 144
YqiW 81 AGQDKEATARARDYFEGYPPSSPSFAILKDGKIMKMVERHEIEGHEPMAVVAKLQEAFEEYCEEV 145
SACOL1464 84 AGQDKEATATMREFI-QQAPSSPSYALFKGQDLVYFMPREFIEGRDINDIAMDLKDAFDENCK-- 145
SACOL1558 81 AGQDKEATQRAREYFEGYAPSSPSFALVKDGKITEMIERHQIEGHDVMNVINQLQTLFNKYCEER 145
SAUSA300_1321 84 AGQDKEATATMREFI-QQAPSSPSYALFKGQDLVYFMPREFIEGRDINDIAMDLKDAFDENCK-- 145
SAUSA300_1463 81 AGQDKEATQRAREYFEGYAPSSPSFALVKDGKITEMIERHQIEGHDVMNVINQLQTLFNKYCEER 145
SERP1006 84 AGQDKEATQTMRDYI-QQVPSSPSYALFKGQHLVHFIPREHIEGRDINDIAMDLKDAFDDNCQ-- 145
SERP1075 81 AGQDKEATQQAREYFEGYAPSSPSFALIKDGKITEMIERHQIEGHDVMDVINQLQALFDKYCEER 145
*

Figure S2

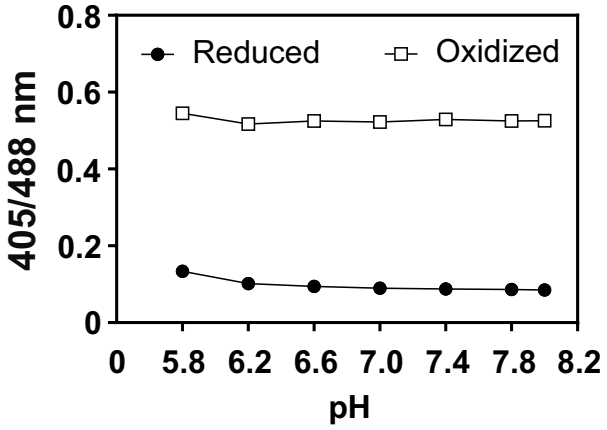


Figure S3

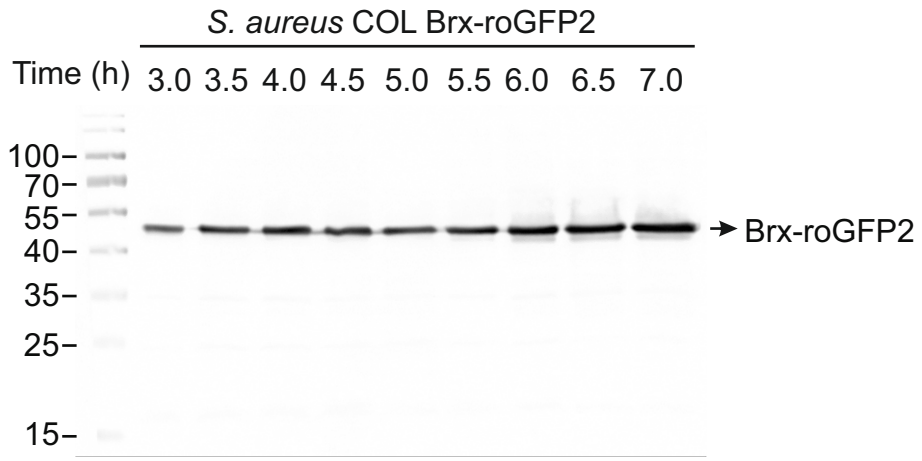

S. aureus COL Brx-roGFP2
Time (h)
3.0 3.5 4.0 4.5 5.0 5.5 6.0 6.5 7.0
100–
70–
55–
40–
35–
25–
15–
Brx-roGFP2

Figure S4

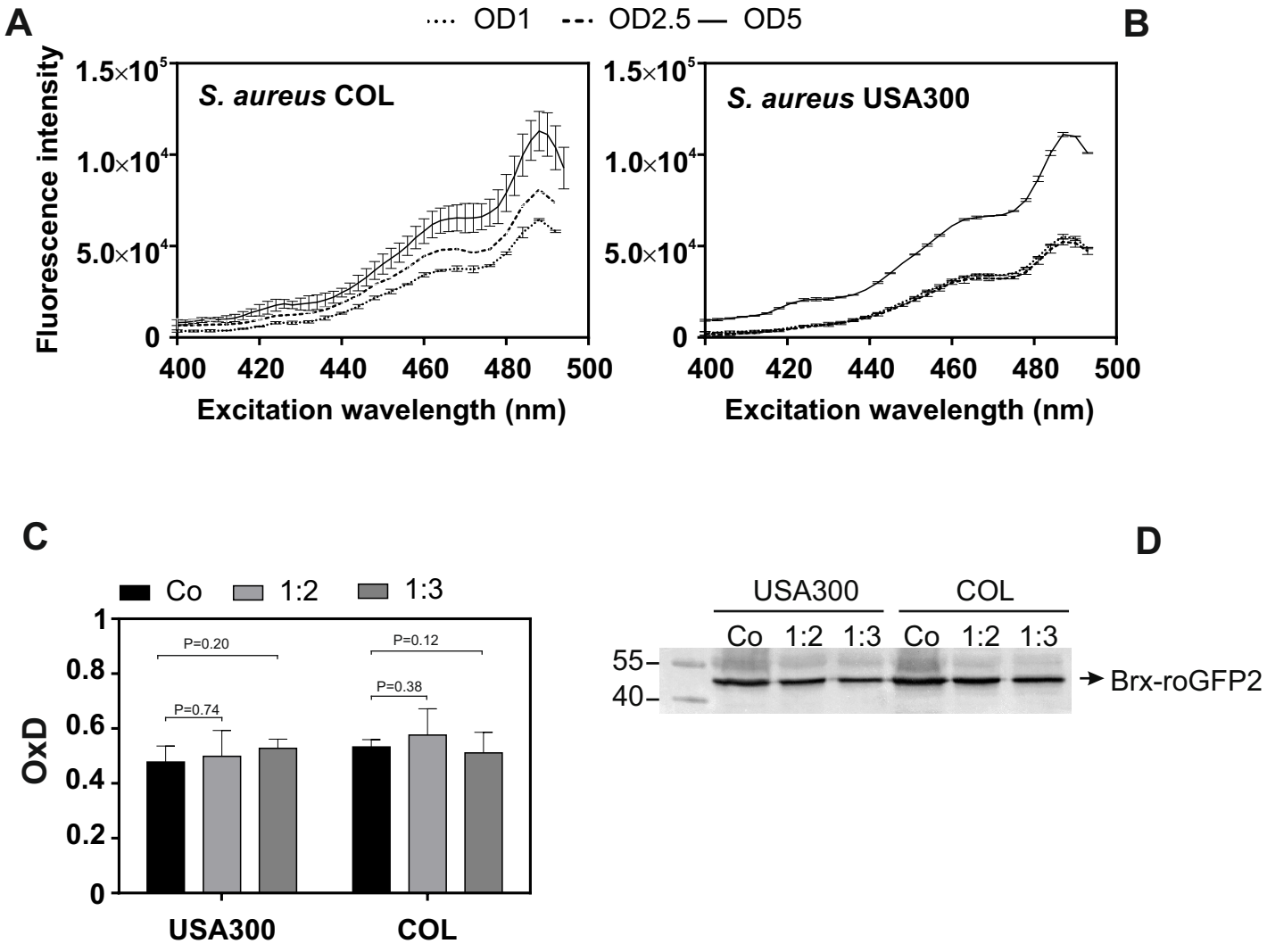
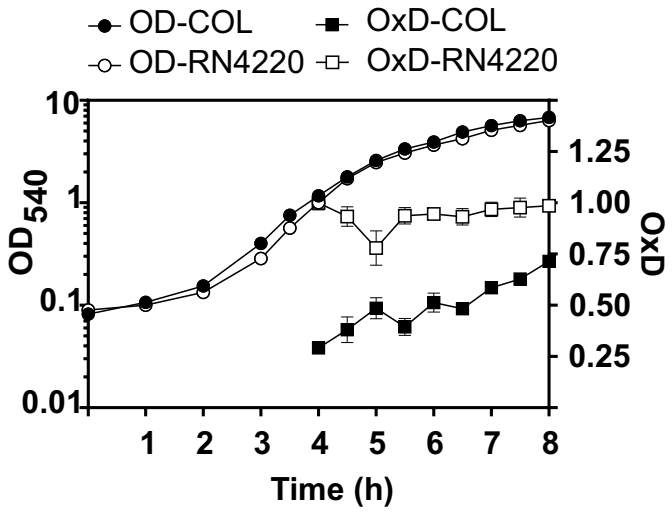

A
OD1
OD2.5
OD5
B
Fluorescence intensity
1.5×10^5
1.0×10^4
5.0×10^4
0
S. aureus COL
S. aureus USA300
400
420
440
460
480
500
Excitation wavelength (nm)
C
Co
1:2
1:3
OxD
1
0.8
0.6
0.4
0.2
0
P=0.20
P=0.74
P=0.12
P=0.38
USA300
COL
D
USA300
COL
Co
1:2
1:3
55–
40–
Brx-roGFP2

Figure S5


OD-COL
OxD-COL
OD-RN4220
OxD-RN4220
10
1
0.1
0.01
OD540
1.25
1.00
0.75
0.50
0.25
OxD
1
2
3
4
5
6
7
8
Time (h)

*

Figure S6

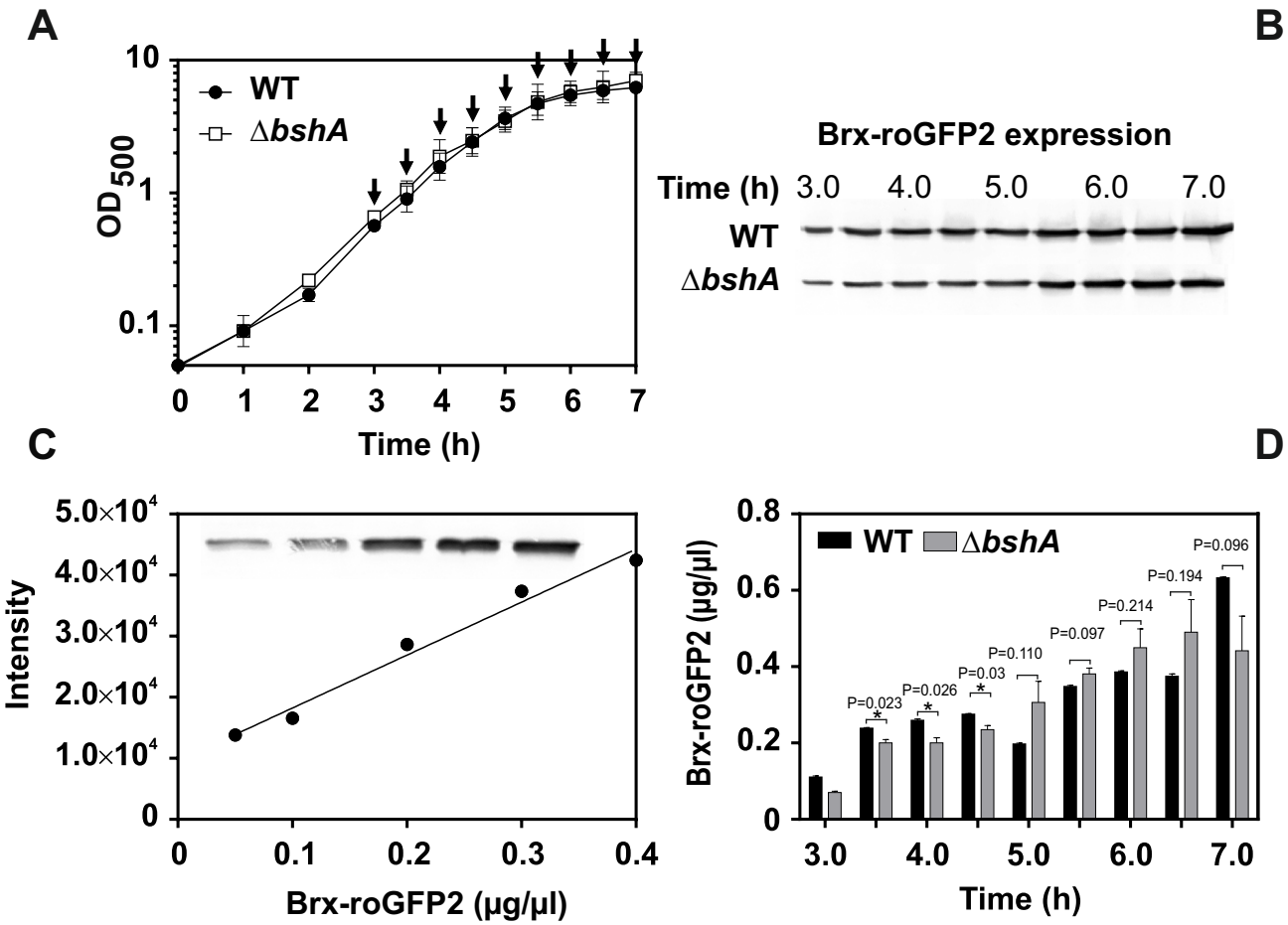

A
B
C
D
WT
ΔbshA
OD500
Time (h)
Brx-roGFP2 expression
Time (h) 3.0 4.0 5.0 6.0 7.0
WT
ΔbshA
Intensity
Brx-roGFP2 (μg/μl)
Brx-roGFP2 (μg/μl)
P=0.023
P=0.026
P=0.03
P=0.110
P=0.097
P=0.214
P=0.194
P=0.096
Time (h)

Figure S7

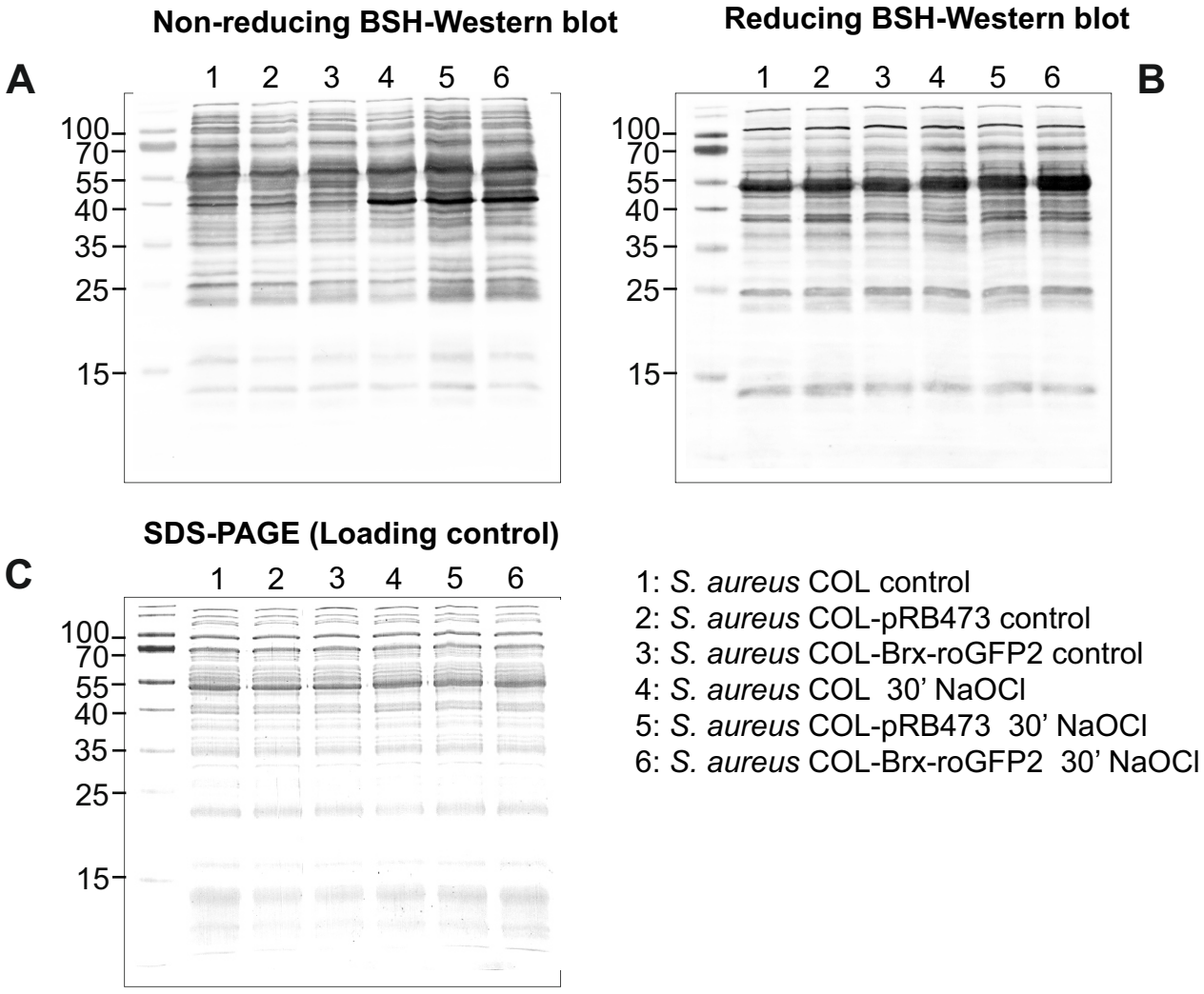

Non-reducing BSH-Western blot
Reducing BSH-Western blot
A
B
C
1 2 3 4 5 6
100
70
55
40
35
25
15
SDS-PAGE (Loading control)
1: S. aureus COL control
2: S. aureus COL-pRB473 control
3: S. aureus COL-Brx-roGFP2 control
4: S. aureus COL 30' NaOCl
5: S. aureus COL-pRB473 30' NaOCl
6: S. aureus COL-Brx-roGFP2 30' NaOCl

Figure S8

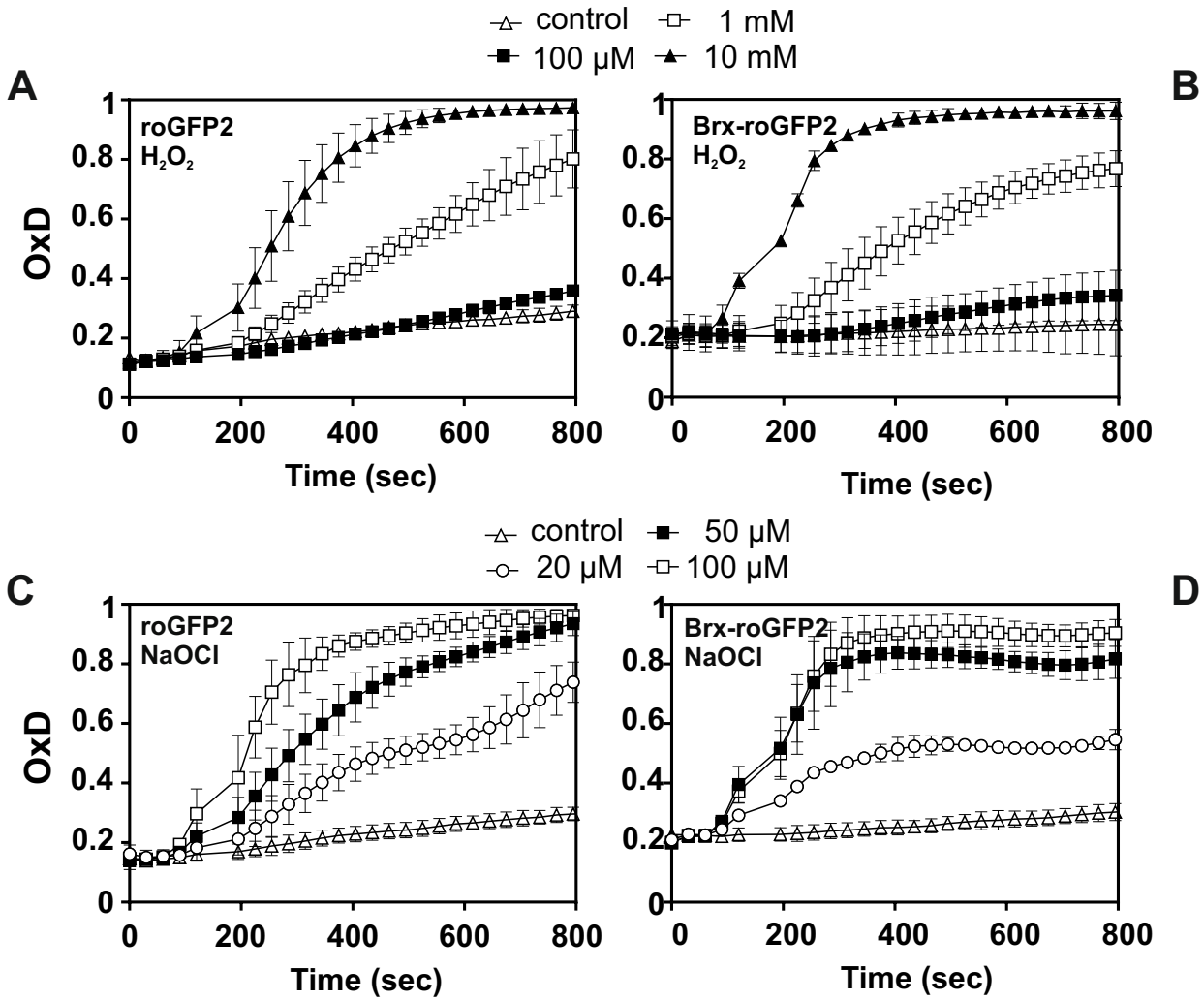

control
1 mM
100 µM
10 mM
A
B
roGFP2
H2O2
Brx-roGFP2
H2O2
OxD
Time (sec)
control
50 µM
20 µM
100 µM
C
D
roGFP2
NaOCl
Brx-roGFP2
NaOCl
OxD
Time (sec)

Table S1: Bacterial strains

Strain	Description	Reference
Escherichia coli		
DH5α	F-φ80d *lacZ* ∆(*lacZYA-argF*) U169 *deoR supE44* ∆*lacU169* (f80*lacZDM15*) *hsdR17 recA1 endA1* (rk- mk+) *supE44 gyrA96 thi1 gyrA69 relA1*	1
BL21(DE3)p*lysS*	F- *ompT hsdS gal* (rb- mb+) DE3(*sam7* ∆*nin5 lacUV5-T7 Gen1*)	1
Staphylococcus aureus		
RN4220	restriction negative strain/ MSSA cloning intermediate derived from 8325-4	2
USA300	CA-MRSA strain	3
COL	Archaic HA-MRSA strain	
COL-∆*bshA*	COL *bshA* mutant	3
USA300-∆*bshA*	USA300 *bshA* mutant	3
COL-Brx-roGFP2	COL pRB473-*brx-roGFP2*	This study
COL-BrxAGC-roGFP2	COL pRB473-*brxAGC-roGFP2*	This study
COL-BrxCGA-roGFP2	COL pRB473-*brxCGA-roGFP2*	This study
COL-BrxAGA-roGFP2	COL pRB473-*brxAGA-roGFP2*	This study
COL-∆*bshA*-Brx-roGFP2	COL *bshA* mutant pRB473-*brx-roGFP2*	This study
USA300-Brx-roGFP2	USA300 pRB473-*brx-roGFP2*	This study
USA300-∆*bshA*-Brx-roGFP2	USA300 *bshA* mutant pRB473-*brx-roGFP2*	This study
RN4220-Brx-roGFP2	RN4220 pRB473-*brx-roGFP2*	This study
Staphylococcus phage 80		4

Table S2: Plasmids

Plasmid	Description	Reference
pQE60-Grx1-roGFP2	pQE60-derivative for overexpression of His-tagged Grx1-roGFP2	5
pET11b	*E. coli* expression plasmid	Novagen
pRB473	pRB373-derivative, *E. coli/ S. aureus* shuttle vector, Amp^r, Cm^r	6
pRB473-XylR	pRB373-derivative, *E. coli/ S. aureus* shuttle vector, containing xylose-inducible P_{Xyl} promoter Amp^r, Cm^r	7
pQE60-Brx-roGFP2	pQE60-derivative for overexpression of His-tagged Brx-roGFP2	This study
pQE60-BrxAGC-roGFP2	pQE60-derivative for overexpression of His-tagged BrxAGC-roGFP2	This study
pQE60- BrxCGA-roGFP2	pQE60-derivative for overexpression of His-tagged BrxCGA-roGFP2	This study
pET11b-roGFP2	pET11b-derivative for overexpression of His-tagged roGFP2	This study
pET11b-Brx-roGFP2	pET11b-derivative for overexpression of His-tagged Brx-roGFP2	This study
pET11b-BrxAGC-roGFP2	pET11b-derivative for overexpression of His-tagged BrxAGC-roGFP2	This study
pET11b-BrxCGA-roGFP2	pET11b-derivative for overexpression of His-tagged BrxCGA-roGFP2	This study
pET11b-BrxAGA-roGFP2	pET11b-derivative for overexpression of His-tagged BrxAGA-roGFP2	This study
pRB473-XylR-Brx-roGFP2	pRB473-derivative expressing Brx-roGFP2 under P_{Xyl}	This study
pRB473-XylR-BrxAGC-roGFP2	pRB473-derivative expressing BrxAGC-roGFP2 under P_{Xyl}	This study
pRB473-XylR-BrxCGA-roGFP2	pRB473-derivative expressing BrxCGA-roGFP2 under P_{Xyl}	This study
pRB473-XylR-BrxAGA-roGFP2	pRB473-derivative expressing BrxAGA-roGFP2 under P_{Xyl}	This study

Table S3. Oligonucleotide primers

Primer name	Sequence (5' to 3')
SAUSA300-1321yphP-FOR-BamHI-NcoI	GTGCCATGGGGATCCATGAATGCATATGATGCTTATATG
SAUSA300-1321yphP-REV-SpeI	GCGACTAGTTTTACAATTTTCGTCAAAGGCA
SAUSA300-1321-yphP-C54A-REV	TAATCCAGCTGCACAGCC**CGC**TGTAGAGTTAATAACTAC
SAUSA300-1321-yphP-C54A-FOR	GTAGTTATTAACTCTACA**GCG**GGCTGTGCAGCTGGATTA
SAUSA300-1321-yphP-C56A-REV	TTGCTAATCCAGCTGC**CGC**GCCGCATGTAGAGTTAATAA
SAUSA300-1321-yphP-C56A-FOR	TAACTCTACATGCGGC**GCG**GCAGCTGGATTAGCAAGACC
1321-roGFP2-FOR-NheI	CTAGCTAGCATGAATGCATATGATGCTTATATGAAAG
1321-brx-C54A56A-REV	TAATCCAGCTGCCGCGCCCGCTGTAGAGTTAATAACTAC
1321-brx-C54A56A-FOR	GTAGTTATTACCTCTACA**GCG**GGC**GCG**GCAGCTGGATTA
roGFP2-REV-BamHI	CGCGGATCCTTAGTGATGGTGATGGTGATGCTTGTACAG CTCGTCCATGC
roGFP2-FOR-NheI	CTAGCTAGCATGGTGAGCAAGGGCGAGGAG
SAUSA300-1321-FOR-BamHI-2	TAGGGATCCGAACAATTTAATTGGAGGAATTAAATATGAA TGCATATGATGCTTATATG
roGFP2-REV-KpnI-3	CGGGGTACCTTACTTGTACAGCTCGTCCATGCCGAG

Restriction sites are underlined and bold bases indicate point mutations

Table S4: Typical Zeiss LSM510meta settings for imaging of Brx-roGFP2 in *S. aureus*

Mode	Channel mode
Imaging setting	
Switch track	line
Track 1	Argon/2 (458, 477, 488 and 514nm)+Transmitted light
Track 2	405nm
Light path	
Track 1+2	Emission filter BP 505-550nm
Acquisition	
Scan mode	Frame
Frame size	212 x 212
Averaging number	4
Scan time	6.15sec
Pixel Dwell	7.73µsec
Bit depth	12bit
zoom	4.4
Channel/ laser setting	
488nm line	
Maximum power	30mW
Laser line attenuator transmission	20%
Pinhole	1AU
Gain	948
405nm line	
Maximum power	30mW
Laser line attenuator transmission	8%
Pinhole	1AU
Gain	948

Table S5: BSH redox potential changes in *S. aureus* COL and *S. aureus* USA300 during growth

***S. aureus* COL**			***S. aureus* USA300**		
Time (h)	**OD540**	**E_{BSH} (mV)**	**Time (h)**	**OD540**	**E_{BSH} (mV)**
3	0.86 ± 0.04	-292.71 ± 4.98	3	1.05 ± 0.05	nd
4	1.97 ± 0.09	-301.60 ± 14.42	4.25	3.61 ± 0.44	-301.16 ± 18.19
4.5	2.72 ± 0.30	-281.44 ± 2.05	5	5.1 ± 0.32	-276.14 ± 10.99
5	3.31 ± 0.26	-280.29 ± 1.94	5.25	5.54 ± 0.05	-274.24 ± 10.72
6	5.2 ± 0.86	-280.12 ± 5.73	6.3	6.2 ± 0.05	-227.88 ± 13.14
7	5.3 ± 0.62	-271.94 ± 4.95	7	6.33 ± 0.04	-235.04 ± 19.18

nd: not determined

Table S6: Effects of antibiotics on the changes of BSH redox potential inside *S. aureus* COL

	E_{BSH} (mV)										
Time (h)	**Control**	**Erythromicin 0.1µM**	**Rifampicin 0.1µM**	**Vancomycin 5µM**	**Ciprofloxacin 0.1µM**	**Gentamicin 0.5µg/ml**	**Ampicillin 10µM**	**Fosfomycin 50nM**	**Lincomycin 5µM**	**Linezolid 2µg/ml**	**Oxacillin 2mM**
0	-286.22 ± 21.21	-282.75 ± 4.48	-280.53 ±6.40	-272.50 ± 1.21	-270.49 ± 4.24	-272.92 ± 4.91	-281.49 ± 1.57	-282.79 ± 4.53	-278.26 ± 4.87	-277.41 ± 3.86	-283.58 ± 0.96
1	-277.47 ± 4.15	-283.48 ± 1.44	-276.81 ± 5.88	-267.38 ± 6.31	-273.69 ± 3.34	-270.55 ± 1.21	-283.29 ± 0.49	-284.07 ± 8.32	-276.47 ± 2.32	-289.84 ± 6.34	-289.69 ±1.77
2	-279.24 ± 1.83	-272.96 ± 8.28	-273.98 ± 2.50	-267.69 ± 3.58	-273.70 ± 1.69	-271.45 ± 2.58	-275.37 ± 5.49	-282.13 ± 4.10	-280.24 ± 7.20	-286.12 ± 4.27	-284.90 ± 0.53
3	-277.10 ± 3.33	-271.00 ± 14.98	-268.47 ± 1.68	-269.00 ± 3.56	-274.00 ± 1.86	-265.97 ± 2.49	-273.14 ± 3.02	-275.65 ± 6.77	-272.51 ± 1.32	-278.92 ± 4.60	-280.22 ± 1.83
4	-265.45 ± 5.74	-244.30 ± 58.37	-267.72 ± 4.90	-255.50 ± 4.72	-267.24 ± 3.35	-269.54 ± 2.78	-263.87 ± 8.47	-269.55 ± 10	-282.31± 1.32	-266.85 ±3.83	-270.80 ± 2.65

Table S7: Response of Brx-roGFP2 in *S. aureus* COL during phagocytosis assays with THP-1 macrophages.

Excitation wavelength (nm)	MFI 100% Reduced	MFI Infected	MFI 100 % Oxidized
405	561.66 ± 21.73	663.11 ± 34.19	655.33 ± 23.35
488	2953 ± 172.98	2252.11 ± 106.06	2095.66 ± 88.69
405/488	0.19 ± 0.01	0.29 ± 0.02	0.31 ± 0.02

THP-1 cells were infected with *S. aureus* COL Brx-roGFP2 for 1 hour at MOI 25, blocked with 10 mM NEM and analyzed by flow cytometry. Emission was measured at 515-545 nm after excitation at 405 and 488 nm. The 405/488 nm ratios of the average mean fluorescence intensities (MFI) of infected macrophages was related to that of fully reduced and oxidized macrophages to calculate the oxidation degree during infection. The Brx-roGFP2 biosensor is 90% oxidized in *S. aureus* COL during infection of THP-1 macrophages. The mean values of 3 experiments are shown, error bars represent the *s.e.m* and P-values were calculated using a Students unpaired two-tailed t-Test by the graph prism software.

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