Supplemental Table 1. Model validation values of Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA) comparisons of ^{1}H-NMR spectra.

Strain (DAP challenge)[a]	Model values[b]		
	R^2	Q^2	CV-ANOVA *p*
Exponential phase (2.5 h)			
616 (-) / 703 (-)	0.9949	0.8093	4.9×10^{-3}
616 (+) / 703 (+)	0.9270	0.8624	4.1×10^{-3}
616 (+) / 616 (-)	0.9506	0.7858	1.8×10^{-3}
703 (+) / 703 (-)	0.8830	0.2326	2.0×10^{-1}
Q2819 (-) / Q2818 (-)	0.8716	0.7034	1.7×10^{-3}
Q2819 (+) / Q2818 (+)	0.9498	0.7033	6.8×10^{-5}
Q2819 (+) / Q2819 (-)	0.8533	0.7535	2.0×10^{-4}
Q2818 (+) / Q2818 (-)	0.7676	0.6805	5.0×10^{-2}
BOY755 (-) / BOY300 (-)	0.9212	0.7565	5.5×10^{-4}
BOY755 (+) / BOY300 (+)	0.9713	0.8063	2.74×10^{-5}
BOY755 (+) / BOY755 (-)	0.9740	0.7768	5.8×10^{-4}
BOY300 (+) / BOY300 (-)	0.9034	0.5650	1.1×10^{-3}
Post-exponential phase (6 h)			
616 (-) / 703 (-)	0.9972	0.9280	1.2×10^{-3}
Q2819 (-) / Q2818 (-)	0.9182	0.7330	1.7×10^{-3}
BOY755 (-) / BOY300 (-)	0.9535	0.8153	1.0×10^{-4}

[a] The metabolomes of Dap^S (616, Q2819, and BOY755) and Dap^R (703, Q2818, and BOY300) strains with (+) or without (-) challenging the cells with DAP were compared by Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA).

[b] R^2 and CV-ANOVA *p* values indicate the degree of model fit, while Q^2 indicates the quality of the model prediction. These values were derived from at least six independent experiments for each group.

Supplemental Table 2. Major contributors to group separation using Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA) of ^{1}H-NMR spectra of daptomycin susceptible and non-susceptible *S. aureus* strains challenged or unchallenged with daptomycin.

Strain (DAP challenge)[a]	Major contributors to class separation[b]			ppm range	Possible metabolites
	616/703	Q2819/ Q2818	BOY755/ BOY300		
Exponential phase (2.5 h)					
Dap^{S} (-) vs. Dap^{R} (-)					
Higher conc. in Dap^{R} (-)	x	x	x	[3.88.. 3.96]	Fructose-6-phosphate
	x	x	x	[3.39 .. 3.43]	Glutamate
	x	x	x	[3.32 .. 3.35]	Fructose-6-phosphate
Lower conc. in Dap^{R} (-)	x	x	x	[4.08 .. 4.14]	Dihydroorotate
	x	x	x	[2.96 .. 3.01]	Asparagine
	x	x		[1.58 .. 1.66]	Arginine/Citrulline
	x		x	[1.52 .. 1.56]	Citrulline
	x		x	[1.90 .. 1.95]	Arginine
Dap^{S} (+) vs. Dap^{R} (+)					
Higher conc. in Dap^{R} (+)	x	x	x	[3.88 .. 3.96]	Fructose-6-phosphate
	x	x		[3.43 .. 3.46]	Glutamate
		x	x	[8.33 .. 8.38]	S-Adenosyl-L-homocysteine
Lower conc. in Dap^{R} (+)	x		x	[4.02 .. 4.05]	Acetyl-CoA
	x		x	[6.00 .. 6.05]	UMP/UDP

	x		x	[3.98 .. 4.02]	Acetyl-CoA
Dap^S (-) vs. Dap^S (+)					
Higher conc. in Dap^S (+)	x	x	x	[5.98 .. 6.05]	UMP/UDP
	x		x	[8.14 .. 8.17]	IMP
	x	x	x	[3.74 .. 3.82]	N-Acetyl-D-glucosamine
	x	x		[2.03 .. 2.06]	N-Acetyl-D-glucosamine
Lower conc. in Dap^S (+)	x	x	x	[8.03 .. 8.06]	n.d.
	x	x	x	[1.58 .. 1.66]	Citrulline
	x	x		[7.94 .. 7.98]	CDP-choline
	x		x	[2.69 .. 2.72]	Aspartate/Malate
	x		x	[2.77 .. 2.83]	Aspartate
Dap^R (-) vs. Dap^R (+)					
Lower conc. in Dap^R (+)	x	x		[2.00 .. 2.03]	N-Acetyl-D-glucosamine
Post-exponential phase (6 h)					
Dap^S (-) vs. Dap^R (-)					
Higher conc. in Dap^R (-)	x	x	x	[3.43 .. 3.46]	Glutamate
	x		x	[2.00 .. 2.03]	N-Acetyl-D-glucosamine
	x		x	[1.66 .. 1.70]	Lysine
	x	x		[2.35 .. 2.41]	Pyruvate
	x		x	[1.40 .. 1.43]	n.d.
	x		x	[1.82 .. 1.87]	Thymine
	x	x		[2.12 .. 2.17]	Acetyl-CoA
	x		x	[1.66 .. 1.70]	Hydroxybutyrate

Lower conc. in Dap^R (-)	x	x	x	[2.77 .. 2.83]	Aspartate
	x	x	x	[2.69 .. 2.72]	Aspartate/Malate
	x	x	x	[2.61 .. 2.66]	Citrate
	x	x	x	[2.82 .. 2.87]	Succinyl-CoA
	x	x	x	[1.95 .. 1.98]	Arginine
	x		x	[3.07 .. 3.12]	Tyrosine
	x	x		[1.97 .. 2.00]	Isoleucine
	x		x	[2.12 .. 2.17]	Glutamate
	x		x	[2.08 .. 2.12]	Glutamate

[a] The metabolomes of Dap^S (616, Q2819, and BOY755) and Dap^R (703, Q2818, and BOY300) strains with (+) or without (-) challenging the cells with DAP were compared by Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA).

[b] Each NMR bin with a high reliability (p(corr)[1] +0.8 or -0.8) and a high magnitude (p[1] > 0.1 or < -0.1) was considered to be a major contributor to the clustering. The ppm regions identified as major contributors for at least two out of the three strain pairs (indicted by "x") and their most likely metabolite annotation guided by the HSQC results of strains 616 and 703 are shown.

Supplemental Table 4. *P* values of Mahalanobis distances analysis on Principle Component Analysis (PCA) 3D scores from ^{1}H-NMR spectra of daptomycin susceptible and non-susceptible *S. aureus* strains with / without daptomycin challenge.

Strain (DAP challenge)[a]	*P* value		
	616/703	Q2819/Q2818	BOY755/BOY300
Dap^S (-) / Dap^S (+)	1.06×10^{-6}	2.79×10^{-6}	2.85×10^{-10}
Dap^S (-) / Dap^R (-)	7.09×10^{-4}	3.52×10^{-5}	3.67×10^{-6}
Dap^S (-) / Dap^R (+)	3.98×10^{-4}	7.48×10^{-6}	1.86×10^{-4}
Dap^S (+) / Dap^R (-)	8.21×10^{-7}	2.43×10^{-6}	4.01×10^{-9}
Dap^S (+) / Dap^R (+)	3.78×10^{-5}	2.11×10^{-8}	3.87×10^{-7}
Dap^R (-) / Dap^R (+)	1.70×10^{-2}	2.23×10^{-3}	1.03×10^{-2}

[a] The metabolomes of Dap^S (616, Q2819, and BOY755) and Dap^R (703, Q2818, and BOY300) strains with (+) or without (-) challenging the cells with DAP were analyzed by PCA. The distance between separated clusters is indicated by *p* values, whereby lower values represent larger distances / larger separation. The *p* values were calculated from at least six independent experiments for each cluster.

Supplemental Figure 1. Growth and pH profiles of daptomycin susceptible (Dap^S) and isogenic derivative non-susceptible (Dap^{NS}) strains. Strain pairs Q2819 / Q2818 (A), BOY755 / BOY300 (B), CB5011 / CB5012 (C), CB5062 / CB5063 (D), and CB1663 / CB1664 (E) were grown in TSB and the optical density and pH were monitored. The results presented are the averages and standard deviations of three independent experiments. Symbols are defined in the figure insets. Statistical significance (p * < 0.1, p ** < 0.05, and p *** < 0.005) of Dap^S versus Dap^{NS} by Student's t test is indicated.

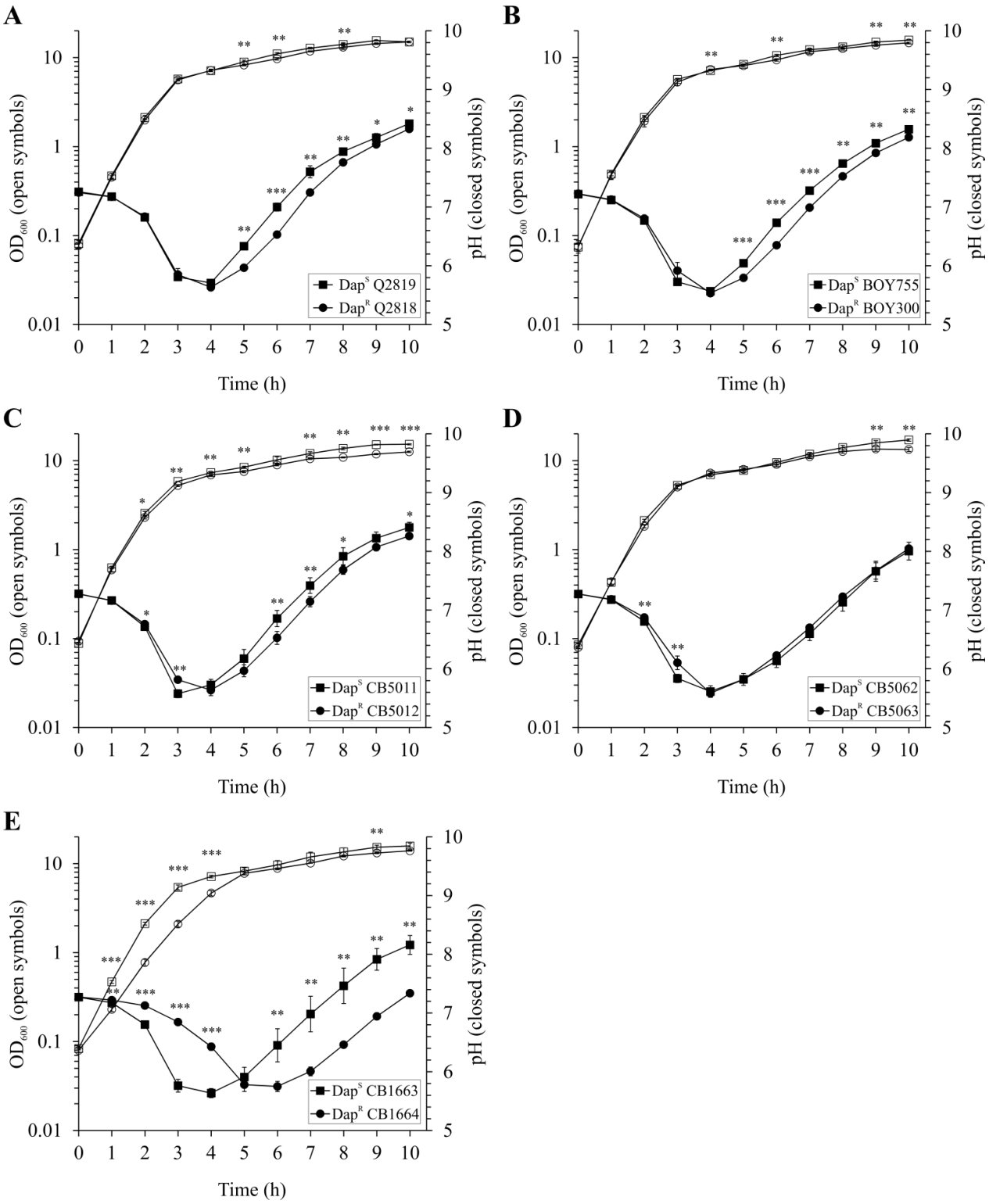
Supplemental Figure 2. Metabolic profiles of daptomycin susceptible (Dap^S Q2819 and BOY755) and isogenic derivative non-susceptible (Dap^{NS} Q2818 and BOY300) strains. Clustering of metabolomes from exponential (2.5h) and post-exponential (6h) growth phase examined by PCA of 1D ^{1}H-NMR spectra and then presented by LDA demonstrates significantly different metabolic profiles in the 2D LDA plots (A and C) and the dendrogram plots (B and D), both of which are generated from the 3D PCA scores. The ellipses correspond to the 95% confidence limits from a normal distribution for each cluster. Each dendrogram node is labeled with a p value; a lower p value indicates a larger separation between the clades. Symbols and colors are defined in the figure.

Supplemental Figure 3. S-plot of Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) of ^{1}H-NMR spectra of daptomycin susceptible (Dap^S 616) and non-susceptible (Dap^{NS} 703) strains from post-exponential (6h) growth phase. The S-plot of OPLS-DA identifies and visualizes the NMR bins which significantly contribute to class separation with high confidence. The X axis cov (t_p,X) provides information about the contribution of the bin with higher absolute values representing higher contributions. The Y axis corr (t_p,X) provides

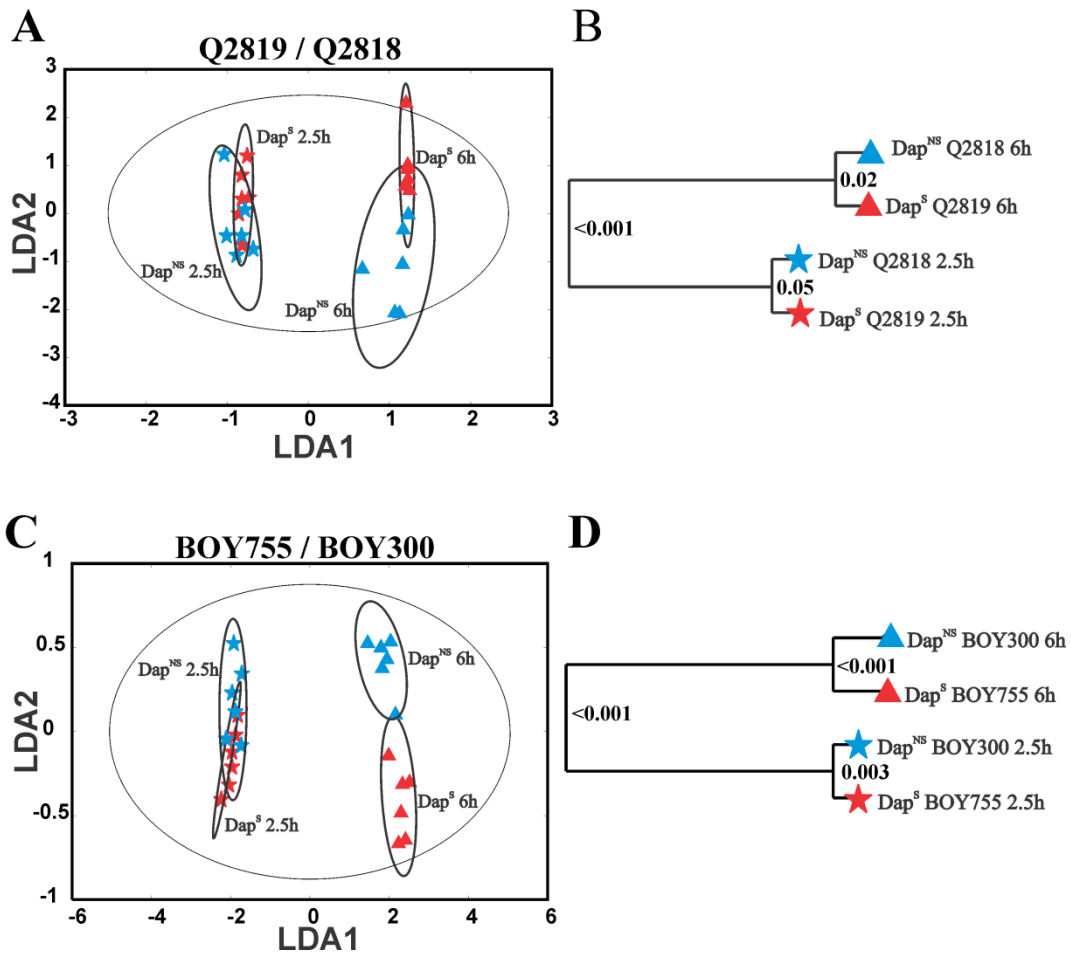
confidence information with higher absolute values representing higher confidence. As example, the bins inside the up-right corner (red square) are significantly decreased in DapNS 703 compared to Dap^S 616, while bins inside the bottom-left corner (green square) are significantly decreased in DapNS 703. Significant bins are assigned to metabolites when possible.

Supplemental Figure 4. Effect of daptomycin challenge on the metabolic profiles of daptomycin susceptible (Dap^S Q2819 and BOY755) and isogenic derivative non-susceptible (Dap^{NS} Q2818 and BOY300) strains. *S. aureus* cells were challenged with daptomycin at concentrations leading to a growth reduction of approximately 50% at the time of harvest after 2.5 h and the intracellular metabolomes were examined by 1D ^{1}H-NMR. Clustering of metabolomes from daptomycin challenged and control cultures was examined by 3D PCA scores of 1D ^{1}H-NMR spectra and then presented by LDA (A and C) and dendrogram plots (B and D). The ellipses correspond to the 95% confidence limits from a normal distribution for each cluster. Each dendrogram node is labeled with a *p* value; a lower *p* value indicates a larger separation between the clades. Symbols and colors are defined in the figure.

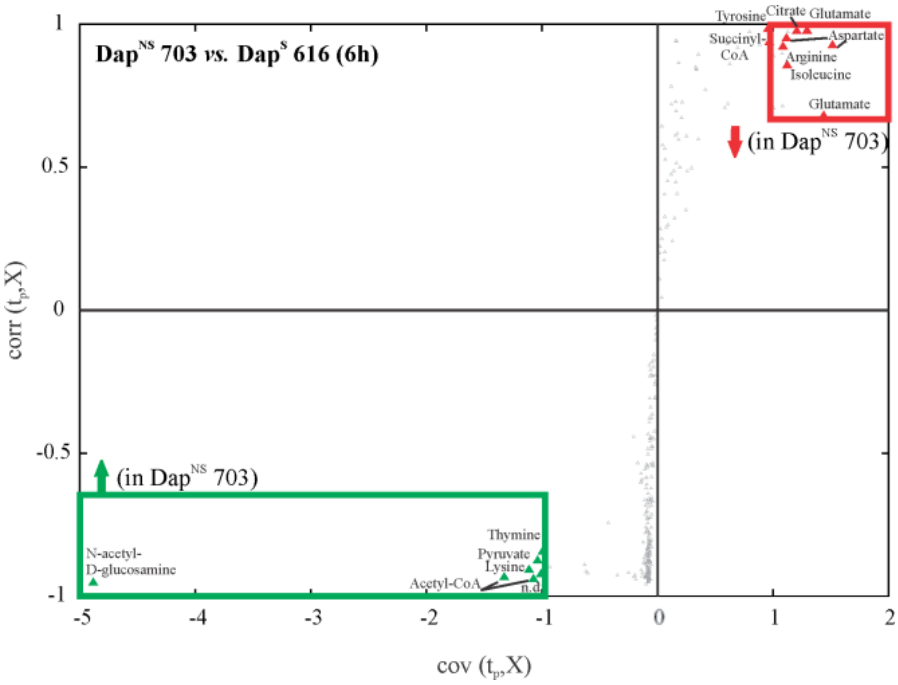
Supplemental Figure 1.



Supplemental Figure 2.



Supplemental Figure 3.



Supplemental Figure 4.