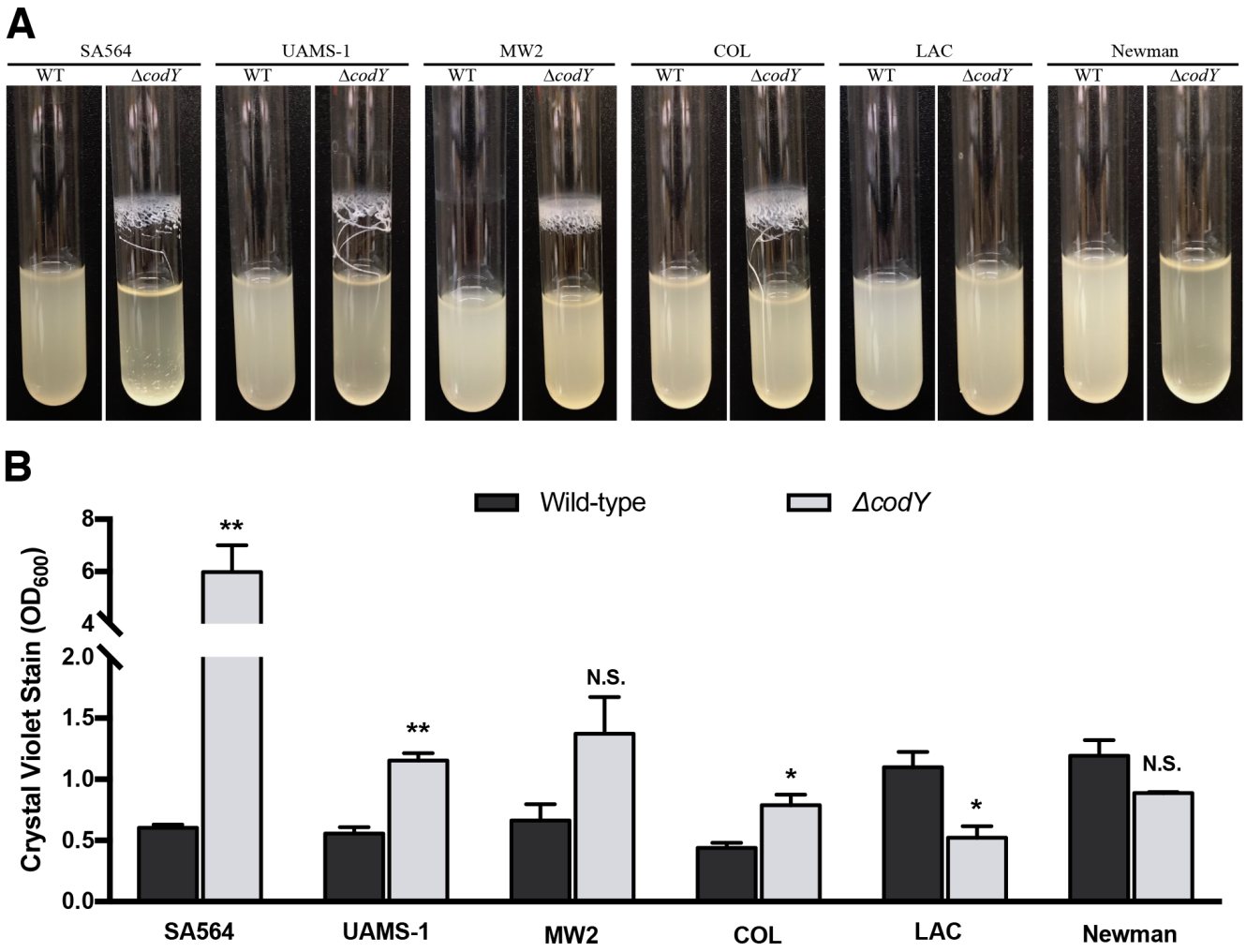
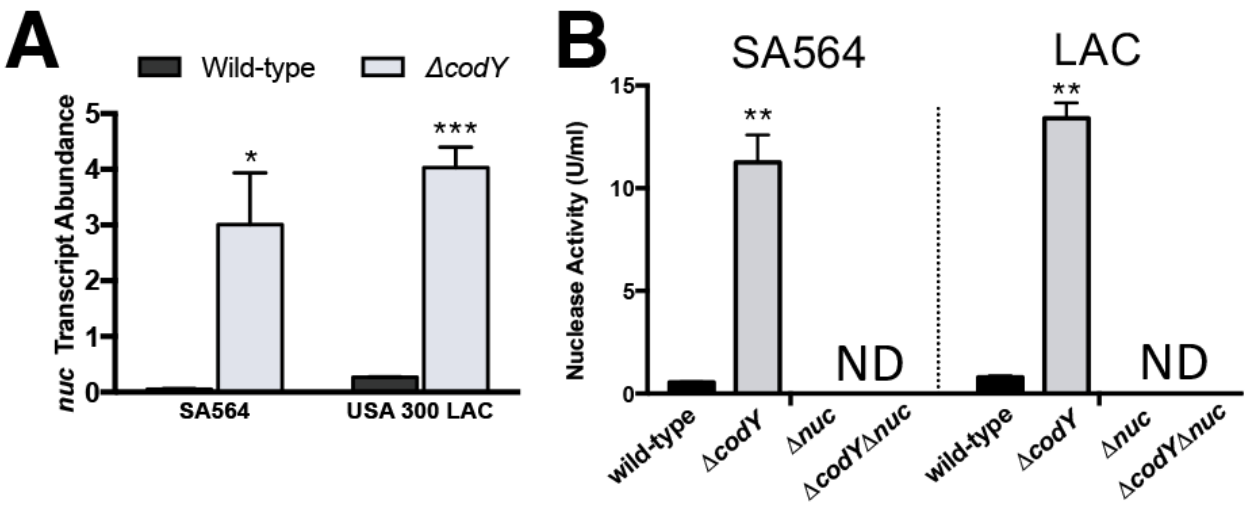
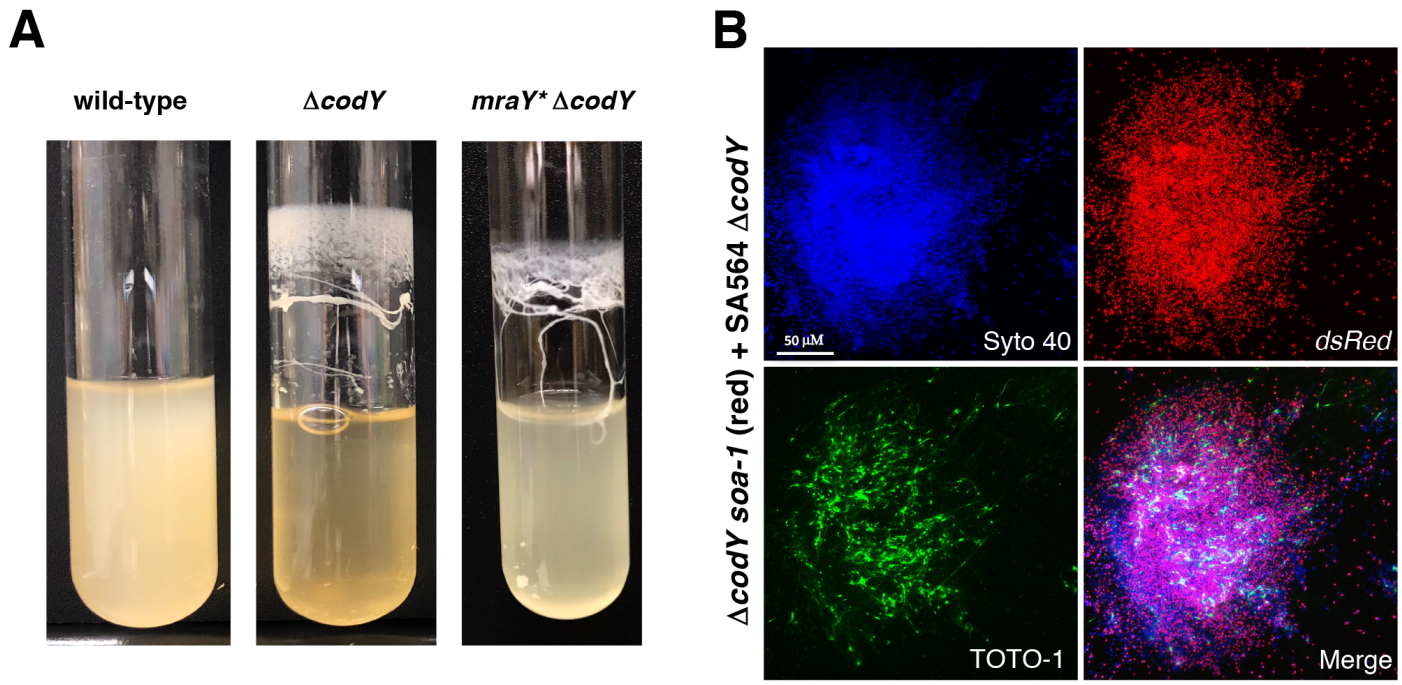


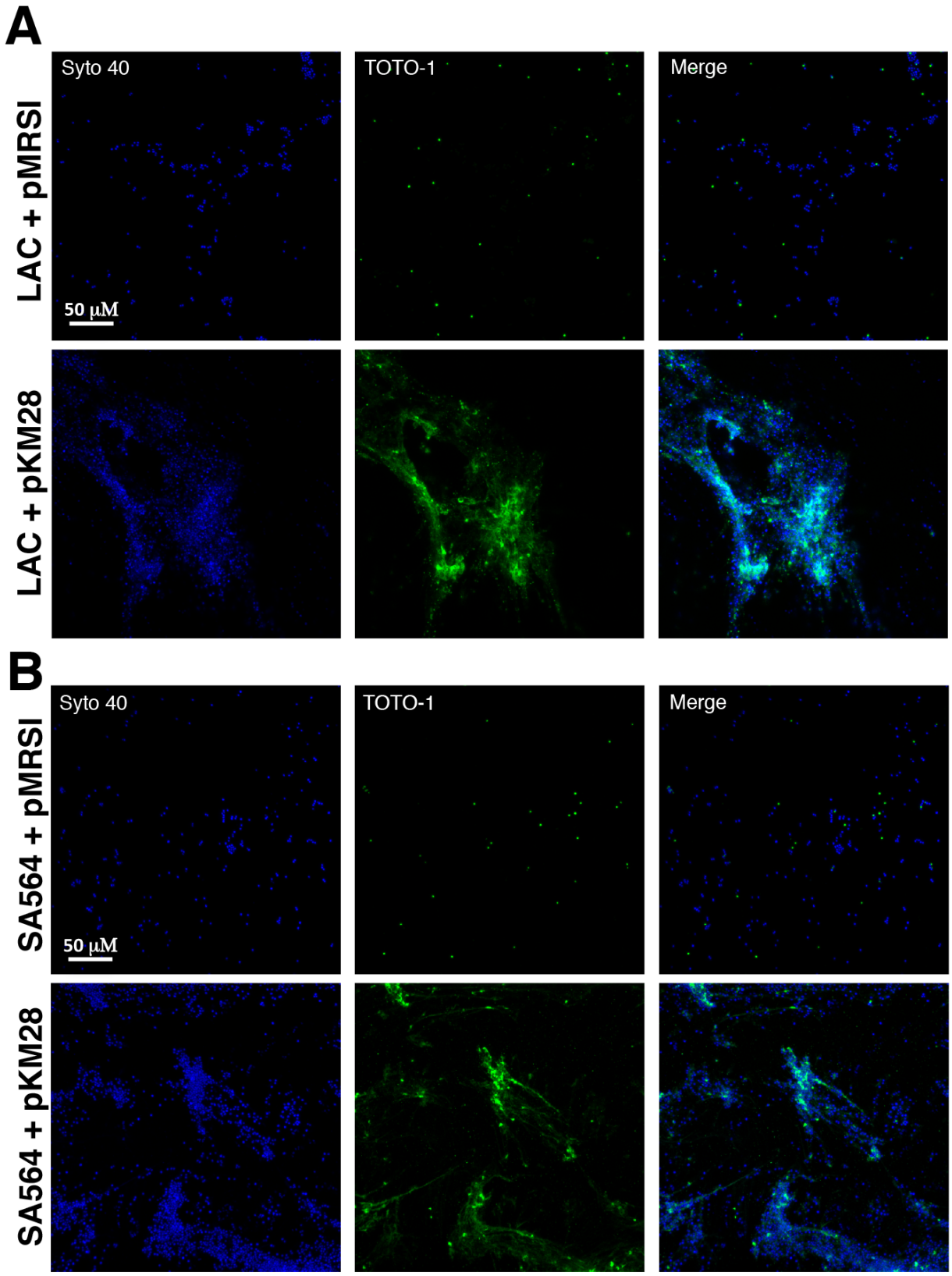
Supplementary Figure 1. Cell aggregation and static biofilm formation are correlated. (A) Representative images from overnight cultures of wild-type and an isogenic Δ*codY* grown in TSB. The experiment was performed multiple times. **(B)** Results from static biofilm assays are shown. Statistics: *$p < 0.05$, **$p<0.01$ compared to the Wild-type parent strain (black bars) using Student's t-test. N.S., not significant.



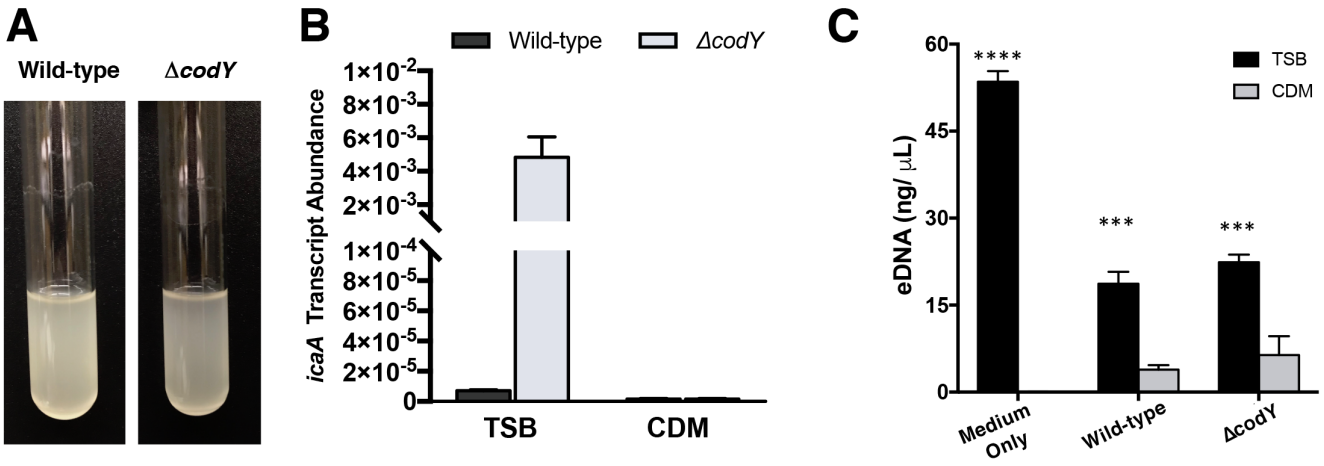
Supplementary Figure 2. Secreted nuclease does not account for the opposing phenotypes observed in Δ*codY* mutants from different isolates. (A) SA564 and LAC cells were grown to exponential phase aerobically in TSB, and *nuc* transcript abundance in wild-type (black) and Δ*codY* mutant (grey) cells was determined by qRT-PCR. Data were normalized to *rpoC* transcript (i.e., copies of *rpoC*). **(B)** Secreted nuclease activity was determined from cultures supernatants during exponential growth in TSB using a FRET assay (see *Materials and Methods*). Nuclease activity was undetectable in Δ*nuc* and Δ*codY* Δ*nuc* mutants. Data indicate the mean ± SEM of at least three independent experiments. *$p < 0.05$, **$p < 0.01$, **$p < 0.001$; Students t-test comparing the Δ*codY* mutant relative to its wild-type. ND, not detected. No statistical difference is observed when Δ*codY* mutants are compared to one another.



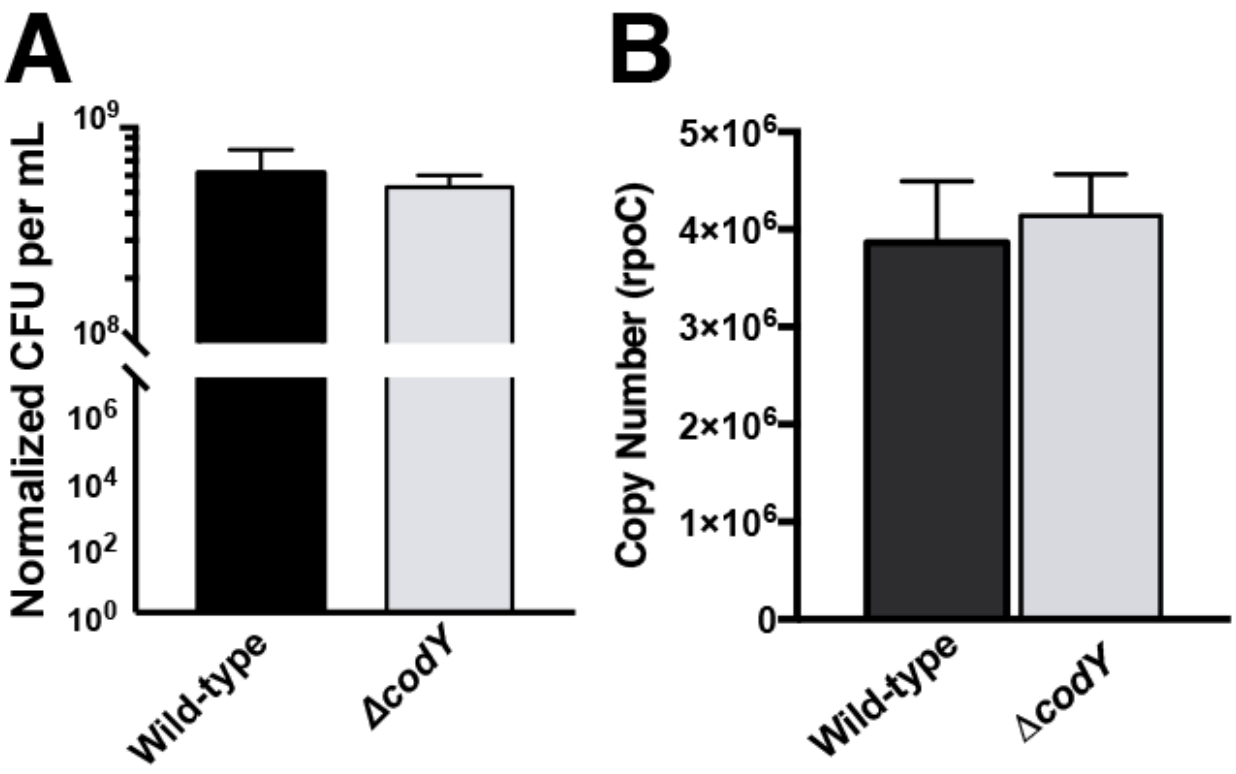
Supplementary Figure 3. A SNP in *mraY* does not account for the loss of aggregation in Δ*codY soa-1*, but co-culturing with a Δ*codY* restores eDNA-dependent cell aggregation. (A) Representative images of overnight cultures in TSB of SA564 (wild-type) as well as the isogenic Δ*codY* mutant and reconstructed *mraY* ΔcodY* mutant. **(B)** Results of a co-culture experiment performed using the suppressor mutant harboring pKM16 (red) and the SA564 Δ*codY* mutant during exponential growth in TSB. All cells are labeled with Syto40 (blue), eDNA and dead cells are labeled with TOTO-1 (green). Images are representative of multiple experiments. Scale bar is 50 μm; all panels are viewed at the same magnification.



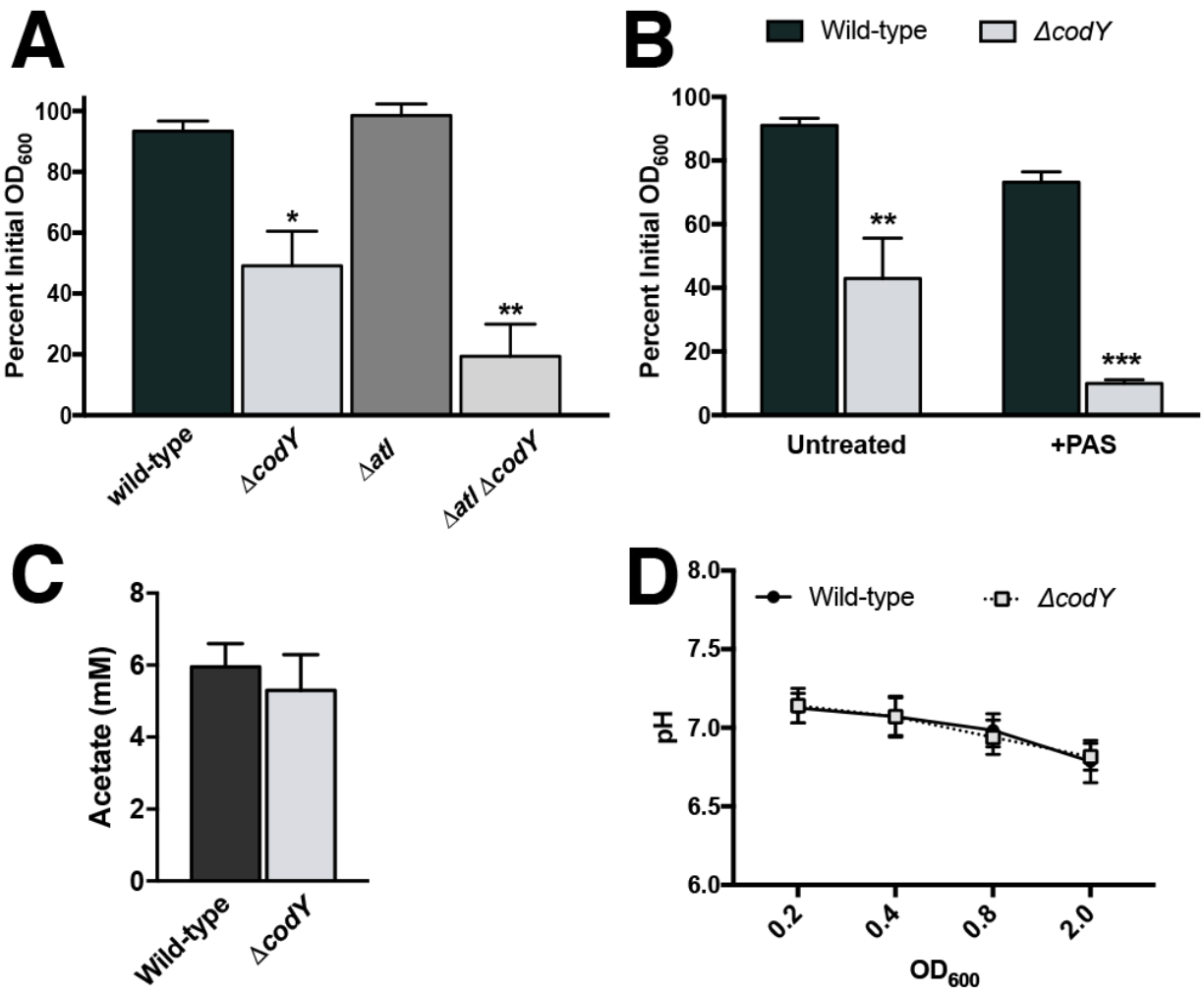
Supplementary Figure 4. Overexpression of *ica* results in eDNA-dependent cell aggregation in LAC wild-type cells. Exponentially growing (**A**) LAC or (**B**) SA564 wild-type cells cultured in TSB harboring pMRSI (vector) or pKM28 ($P_{sarA\text{-}P1}$-*icaADBC*) cells were imaged using CSLM. All cells were visualized using Syto 40 (blue signal) while eDNA and dead cells were stained by TOTO-1 (green signal). All images are representative of multiple experiments. Scale bar: 10 μm where indicated; all panels are viewed at the same magnification.



Supplementary Figure 5. ***ΔcodY* mutants fail to form cell aggregates in CDM medium. (A)** Representative images of the SA564 wild-type and Δ*codY* mutant cultured overnight in CDM medium. **(B)** SA564 was grown to exponential phase aerobically in TSB or CDM, and *icaA* transcript abundance in wild-type (black) and a ΔcodY mutant (grey) cell was determined by qRT-PCR. Data were normalized to *rpoC* transcript. **(C)** eDNA was extracted from media only or supernatants from exponentially growing cells in TSB or CDM and quantified. ***$p < 0.001$, ****$p < 0.0001$; Two-way ANOVA comparing TSB to the CDM for each condition with Sidak's multiple comparison post hoc test.



Supplementary Figure 6. Cell lysis is not increased in a Δ*codY* mutant. SA564 (black bars) and Δ*codY* mutant cells (grey bars) were grown exponential phase aerobically in TSB. (**A**) Colony forming units were determined by dilution plating. (**B**) DNA was extracted from culture supernatants and *rpoC* copy number was determined using quantitative PCR.



Supplementary Figure 7. Atl is not required for cell aggregation in a Δ*codY* mutant. (A) Results of a settling assay are shown for cells of the indicated SA564 strains grown in TSB or (B) for the indicated SA564 strains in the presence or absence of polyanthole sulfate (PAS). Statistics: (A) *$p <$ 0.05, *$p < 0.05$, **$p<0.01$, ANOVA with Dunnett's post analysis compared to Wild-type (B) **$p<0.01$, ***$p <0.001$ compared to Wild-type (black bars) using Student's t-test. **(C)** Acetate concentration and **(D)** culture pH were determined from culture supernatants during exponential phase, when Δ*codY* mutant cells aggregate. No statistical difference was found using a two-tailed t-test.

Table S1. Image analysis of Figure 4B.

Raw	**Aggregate**	**Dispersed**
Syto 40	25684	1779
dsRed (wt)	3314	642

Normalized	Aggregate	Dispersed
Syto 40	22371	1137
dsRed (wt)	3314	642
Fold-enrichment (Δ*codY*)	**6.8**	**1.8**

Pixel intensities for the regions of interest are shown

Table S2. Bacterial strains used in this study.

S. aureus strains	Relevant genotype or description	Source or Reference[a]
RN4220	restriction deficient, highly transformable	(1)
SRB337	USA200 MSSA UAMS-1	(2)
SRB547	Newman MSSA Isolate	(3)
SRB561	SRB547 Δ*codY::ermC*	
SRB687	USA300 LAC CA-MRSA Em^S	(4)
SRB813	SRB337 *codY::*ϕNΣ *(erm::tetM)*^{B}	(5)
SRB892	SRB687 *codY::*ϕNΣ *(erm::tetM)*	
SRB1211	SA546 MSSA Isolate	(6)
SRB1218	SRB1211	
SRB1233	SRB687 *nuc*::ϕNΣ	
SRB1234	SRB687 *codY*::ϕNΣ*(erm::tetM) nuc*::ϕNΣ	
SRB1236	SRB1211 *nuc*::ϕNΣ	
SRB1237	SRB1211 *codY*::ϕNΣ*(erm::tetM) nuc*::ϕNΣ	
SRB1243	SRB1211 *codY*::ϕNΣ*(erm::tetM) soa-1* ($MraY^{L113M}$, *icaB* Q223*)	
SRB1276	COL MRSA Isolate	(7)
SRB1277	SRB1276 Δ*codY::ermC*	
SRB1278	MW2 MRSA Isolate	(8)
SRB1279	SRB1278 Δ*codY::ermC*	
SRB1345	SRB1211 / pKM15	
SRB1346	SRB1211 *codY*::ϕNΣ (*erm::tetM*) / pKM15	
SRB1347	SRB687 *codY*::ϕNΣ (*erm::tetM*) / pKM15	
SRB1351	SRB1211 / pKM16	
SRB1352	SRB1211 *codY*::ϕNΣ (*erm::tetM*) / pKM16	
SRB1353	SRB1211 *codY*::ϕNΣ (*erm::tetM*) *soa-1* / pKM16	
SRB1378	SRB1276 Δ*codY*::ermC / pKM16	
SRB1379	SRB687 *codY*::ϕNΣ (*erm::tetM*) / pKM16	
SRB1468	SRB1211 Δ*ica::tetM*	
SRB1472	SRB1211 Δ*ica::tetM* Δ*codY::ermC*	
SRB1507	SRB1211 *mraY**(L113M)	
SRB1517	SRB1211 *mraY**(L113M) Δ*codY::ermC*	
SRB1575	SRB1211 *codY*::ϕNΣ (*erm::tetM*) *soa-1* / pCN51	
SRB1576	SRB1211 *codY*::ϕNΣ (*erm::tetM*) *soa-1* / pKM26	
SRB1580	SRB687 / pKM28	
SRB1581	SRB1211 / pKM28	
SRB1583	SRB687 / pMRSI	
SRB1584	SRB1211 / pMRSI	
SRB1603	SRB1211 / pKK30	
SRB1604	SRB1211 codY::ϕNΣ (*erm::tetM*) / pKK30	
SRB1605	SRB1211 *codY*::ϕNΣ (*erm::tetM*) / pKM25	
SRB1618	SA113 Δ*lgt*::ermB	(9)

SRB1624	SRB1211 ∆*lgt*::ermB	
SRB1625	SRB1211 Δ*lgt::ermB codY*::φNΣ (*erm::tetM*)	
P. aeruginosa **Strains**		
SRB1611	PA01	A. Oglesby-Sherrouse

[a] Unless noted otherwise strains were constructed during the course of this study.
[b] Resistance marker was exchanged using the NTML toolbox as described in (10).

Table S3. Oligonucleotides used in this study.[a]

Primer	Sequence (5' to 3')[b,c]	Purpose
oDS001	CGAAAGAACAATACGCAAAGAGG	qRT-PCR of *nuc*
oDS002	TGCATTTGCTGAGCTACTTAGA	qRT-PCR of *nuc*
oKM74	CCGCGCATGCTATTTTTGACTAAACCAAATGC	Construction of pKM15
oKM75	AAGGAATTCTGCTCGATACATTTGCCCGATAA	Construction of pKM15
oKM76	AAAGGATCCTGCTCGATACATTTGCCCGAT	Construction of pKM16
oKM77	GGCGGTCGACTATTTTTGACTAAACCAAATGC	Construction of pKM16
oKM102	GCCCGAATTCAATCATTAGAACCAGTTACTATTGG	Construction of pKM22
oKM103	TTGGTCGACTTTACCATTTGTACCCGTTACAGC	Construction of pKM22
oKM111	*TCTCTCGTTTTAGATAATAAGCTCAT*TACATTTACCCTCCATCAATAAG	Construction of pKM25
oKM112	*ATGAGCTTATTATCTAAAACGAGAGA*	Construction of pKM25
oKM113	TTTGGATCCTTATTTACTTTTTTCTAATTCATC	Construction of pKM25
oKM120	AATTAAAGCGGCCGCTAATGACTTTCTAAATGGAAAATAC	Construction of pKM25
oKM121	GGGGCATGCCTATTTTTGACTAAACAAAATGCTAACC	Construction of pKM26
oKM122	*CACATGTAATTCCTCCTTTTATAGATTATC*TGGATCCTGCTCGATACATTTGCCCGATA	Construction of pKM26
oKM123	*GATAATCTATAAAAGGAGGAATTACATGTG*AAGTATAGAAAATTTATAATTTTAG	Construction of pKM26
oKM124	CCCGAATTCCTAATCTTTTTCATGGAATCCGTCCC	Construction of pKM26
oKM125	GGCAGATCTAGATAATCTATAAAAGGAGGAATTACATTTGCAATTTTTTAACTTTTTGCT	Construction of pKM28
oKM126	GCCGCATGCTTAATAAGCATTAATGTTCAATTTATATGG	Construction of pKM28
oNW039	ACCGGCAACTGGGTTTATT	qRT-PCR of *icaB*
oNW040	TGCATATCGTGGGTATGTGTT	qRT-PCR of *icaB*
oNW043	TGAACAAGAAGCCTGACATAAA	qRT-PCR of *icaA*
oNW044	CGTATTTGAGTGCAAGAACATTAG	qRT-PCR of *icaA*
oSRB239	GGATTGGCTTCACCTGAAAA	qRT-PCR of *rpoC*
oSRB240	CTTTCACGACGTACTTTAGA	qRT-PCR of *rpoC*
FRET substrate	5Cy3-CCCGGATCCACCCC-3BHQ_2	DNA FRET assay substrate

[a] oKM primers were designed using SA564 as a reference genome (GenBank: CP010890.1). All others were designed from MRSA252.

[b] Underline denotes the presence of a restriction site for cloning.

[c] Italics denotes sequence homology for use in overlapping PCR.

Table S4. Plasmids used in this study.

Plasmid	Relevant Genotype	Reference[a]
pCN51	Shuttle vector for use in Gram positives (Ap^R, Em^R)	(11)
pJB38	Temperature sensitive plasmid for alleleic exchange (Ap^R, Cm^R)	(10)
pKK30	Stably mainted shuttle vector for *S. aureus* (Ap^R, Tm^R)	(12)
pKM15	pMRSI with SarA-P1 promoter for constituative sGFP expression (Ap^R, Cm^R)	
pKM16	pMRSI with SarA-P1 promoter for constituative sDsRed expression (Ap^R, Cm^R)	
pKM22	pJB38 with *mraY** allele ($MraY^{L113M}$) (Ap^R, Cm^R)	
pKM25	pKK30 with native promoter fused to CodY for complementation (Ap^R, Tm^R)	
pKM26	pCN51 with SarA-P1 promoter fused to *icaB* with TIR ribosome binding site (Ap^R, Em^R)	
pKM28	pMRSI with SarA-P1 promoter fused to the *icaADBC* locus (Ap^R, Cm^R)	
pMRSI	sGFP-sDsRed double reporter shuttle vector (Ap^R, Cm^R)	(5)

[a]Unless otherwise noted all plasmids were engineered during this study.

References

1. Schenk S, Laddaga RA. 1992. Improved method for electroporation of *Staphylococcus aureus*. FEMS Microbiol Lett 73:133-8.
2. Gillaspy A, Hickmon S, Skinner R, Thomas J, Nelson C, Smeltzer M. 1995. Role of the accessory gene regulator (*agr*) in pathogenesis of staphylococcal osteomyelitis. Infect Immun 63:3373-3380.
3. Duthie ES, Lorenz LL. 1952. Staphylococcal coagulase; mode of action and antigenicity. J Gen Microbiol 6:95-107.
4. Boles BR, Thoendel M, Roth AJ, Horswill AR. 2010. Identification of genes involved in polysaccharide-independent *Staphylococcus aureus* biofilm formation. PLoS One 5:e10146.
5. Waters NR, Samuels DJ, Behera RK, Livny J, Rhee KY, Sadykov MR, Brinsmade SR. 2016. A spectrum of CodY activities drives metabolic reorganization and virulence gene expression in *Staphylococcus aureus*. Mol Microbiol 101:495-514.
6. J.M. M, R.K. S. 1990. Genetic analysis of natural populations of *Staphylococcus aureus*, p 59-67. *In* Novick RP (ed), Molecular Biology of the staphylococci. VCH Publishers, New York, NY.
7. Dyke K, Jevons M, Parker M. 1966. Penicillinase production and intrinsic resistance to penicillins in *Staphylococcus aureus*. Lancet 1:835-838.
8. Baba T, Takeuchi F, Kuroda M, Yuzawa H, Aoki K, Oguchi A, Nagai Y, Iwama N, Asano K, Naimi T, Kuroda H, Cui L, Yamamoto K, Hiramatsu K. 2002. Genome and virulence determinants of high virulence community-acquired MRSA. Lancet 359:1819-1827.
9. Stoll H, Dengjel J, Nerz C, Götz F. 2005. *Staphylococcus aureus* deficient in lipidation of prelipoproteins is attenuated in growth and immune activation. Infect Immun 73:2411-2423.
10. Bose JL, Fey PD, Bayles KW. 2013. Genetic tools to enhance the study of gene function and regulation in Staphylococcus aureus. Appl Environ Microbiol 79:2218-24.
11. Charpentier E, Anton AI, Barry P, Alfonso B, Fang Y, Novick RP. 2004. Novel cassette-based shuttle vector system for gram-positive bacteria. Appl Environ Microbiol 70:6076-6085.
12. Krute CN, Krausz KL, Markiewicz MA, Joyner JA, Pokhrel S, Hall PR, Bose JL. 2016. Generation of a stable plasmid for in vitro and in vivo studies of Staphylococcus. Appl Environ Microbiol doi:10.1128/AEM.02370-16.