SUPPLEMENTAL MATERIALS

Minimal Peptidoglycan Turnover in Wild-Type and PG Hydrolase and Cell Division Mutants of *Streptococcus pneumoniae* D39 Growing Planktonically and In Host-Relevant Biofilms

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TABLE S1. Bacterial strains used in this study

TABLE S2. Oligonucleotide primers used in this study (order follows Table S1)

SUPPLEMENTAL FIGURE LEGENDS

REFERENCES FOR SUPPLEMENTAL MATERIALS

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TABLE S1. *Streptococcus pneumoniae* strains used in this study[a]

Strain number	Genotype (description)[b]	Antibiotic Resistance[c]	Reference or source
EL59	R6	None	(1)
IU1690	D39	None	(1)
IU1824	D39 Δ*cps rpsL1*	Str^R	(1)
IU1868	TIGR4	None	(2)
IU1945	D39 Δ*cps*	None	(1)
IU2336	D39 Δ*cps kan*-t1t2-P_c-*pcsB*$^+$	Kan^R	(3)
IU3766	D39 Δ*lytA*<>P_c-*aad9*	$Spec^R$	(4)
IU3875	D39 Δ*cps* Δ*pmp23*<>P_c-*kan*	Kan^R	(4)
IU3877	D39 Δ*cps* Δ*lytB*<>P_c-*kan-rpsL*$^+$	Kan^R	(4)
IU3880	D39 Δ*cps* Δ*dacB*<>P_c-*kan-rpsL*$^+$	Kan^R	(4)
IU3881	D39 Δ*cps* Δ*spd_0703*<>*kan*	Kan^R	(4)
IU3900	D39 Δ*cps* Δ*lytA*<>P_c-*aad9*	$Spec^R$	(4)
IU5648	Δ*cps rpsL1 divIVA*$^+$-P_c-*kan-rpsL*$^+$	Kan^R	H.-C. Tsui (unpublished)
IU5661	D39 Δ*cps rpsl1* Δ*divIVA* (IU5648 transformed with markerless Δ*divIVA* amplicon)	Str^R	This study
IU6647	D39 Δ*cps* Δ*pbp1a*::P_c-*erm* (IU1945 transformed with Δ*pbp1a*::P_c-*erm* amplicon)	Erm^R	This study
IU7204	D39 Δ*cps* Δ*dacA*:: P_c-*kan-rpsL*$^+$ (IU1945 transformed with Δ*dacA*:: P_c-*kan-rpsL*$^+$ amplicon)	Kan^R	This study
IU9086	Δ*cps rpsL1* Δ*mapZ*::P_c-*kan-rpsL*$^+$ (IU1824 transformed with Δ*mapZ*::P_c-*kan-rpsL*$^+$ amplicon)	Kan^R	A. Perez (unpublished)
IU9175	D39 Δ*cps rpsL1* Δ*mapZ* (IU9086 transformed with markerless Δ*mapZ* amplicon)	Str^R	This study
IU9709	D39 Δ*cps* Δ*murMN*::P_c-*erm* (IU1945 transformed with Δ*murMN*::P_c-*erm* amplicon)	Erm^R	This study
K270	D39 Δ*cps* Δ*pgdA*::P_c-*kan-rpsL*$^+$ (IU1945 transformed with Δ*pgdA*::P_c-*kan-rpsL*$^+$ amplicon)	Kan^R	This study
K447	D39 Δ*cps* Δ*adr*::P_c-*kan-rpsL*$^+$ (IU1945 transformed with Δ*adr*::P_c-*kan-rpsL*$^+$ amplicon)	Kan^R	This study

[a]Strains were constructed and characterized as described in Materials and Methods.

[b]Primers used to synthesize fusion amplicons are listed in Table S2.

[c]Antibiotic resistance markers: Erm^R, erythromycin; Kan^R, kanamycin; $Spec^R$, spectinomycin; Str^R, streptomycin.

TABLE S2. Oligonucleotide primers used in this study (order follows Table S1)

Primer	Sequence (5' to 3')	Template[a]	Amplicon Product
For construction of IU5661 (Δ*cps* Δ*divIVA*)			
LIIR-015	TGGATAAAGAAGGTAGAAGATAGCTATGCTC	D39	5' upstream fragment
SC238	TAACCGTCCAGTTATTATTAAGTAAGTGATAGC TCCAGTGCATCCGACAGGTCCAAC		
SC237	CCGTCCAGTTATTATTAAGTAAGTGATAGCTCC AGTGCACCGACAGGTCCAACACCAG	D39	3' downstream fragment
LIIF-013	CACGTTGGACATGCTATGAACAAGATT		
For construction of IU6647 (Δ*cps* Δ*pbp1a*::Pc-*erm*)			
P234	CCCTTGTGTTCATAGCGAGGATAAGCA	D39	5' upstream fragment with 60nt of 5' *pbp1a*
P236	CATTATCCATTAAAAATCAAACGGATCCTACAAGC TTAAGAAGCTAATGCTCAGATACTT		
Erm Forward	ATGAACAAAAATATAAAATATTCTCAAAACTTT	P_c-*erm* cassette[b]	P_c-*erm* cassette
Erm reverse	TTATTTCCTCCCGTTAAATAATAGATAACTAT		
P237	CAAAAGCATAAGGAAAGGGGCCCCAACAATCAAA TACAACCCCTGATCAACAAAATC	D39	60 nt of 3' *pbp1a* and 3' downstream fragment
P235	AGGCAAGCCTGCAACCATGGTCTTGAAA		
For construction of IU7204 (Δ*cps* Δ*dacA*::P_c-*kan-rpsL*$^+$)			
P150	AGCCTGCAATATGCAAGCGATCCCTCTT	D39	5' upstream fragment with 60nt of 5' *dacA*
P152	CATTATCCATTAAAAATCAAACGGATCCTAAGCAG TAGAAACACCCCCTAAAAGAGAGAC		
Kan rpsL forward	TAGGATCCGTTTGATTTTTAATGGATAATG	P_c-*kan-rpsL*$^+$ cassette[b]	P_c-*kan-rpsL*$^+$ cassette
Kan rpsL reverse	GGGCCCCTTTCCTTATGCTTTTG		
P153	CAAAAGCATAAGGAAAGGGGCCCTTCTTCTTAAAA GTTTGGTGGAATCAGTTTG	D39	60 nt of 3' *dacA* and 3' downstream fragment
P151	TATCGTTGATGAGGGAGCAAGCGTCCACTA		
For construction of IU9175 (Δ*cps* Δ*mapZ*)			
P1523	GAGGTCTCTATTCTCAAAGATGTGGCAACTGTC	D39	5' upstream fragment
AJP92	CCGACAAAGTAGCCTGTCTTACAATCAAATTGCGG TTCTTGAGCTTCT		
AJP93	GCTCAAGAACCGCAATTTGATTGTAAGACAGGCTA CTTTGTCGGAAATGGC	D39	3' downstream fragment
P1526	AATTGCATATCACCGTACTCAATACCATTGTG		

For construction of IU9709 (Δ*cps* Δ*murMN*::P-erm)			
P1535	GGGTGAATGTCCTCTACCCTGATGCCAATC	D39	5' upstream fragment with 60nt of 5' *murMN*
P1536	ACATTATCCATTAAAAATCAAACGGATCCTAGGCT AATTCATGT TCTTTGACAAACTGAT		
Erm Forward	ATGAACAAAAATATAAAATATTCTCAAAACTTT	P-*erm* cassette[b]	P-*erm* cassette
Erm reverse	TTATTTCCTCCCGTTAAATAATAGATAACTAT		
P1537	CAAAAGCATAAGGAAAGGGGCCCCATCCATCTCC TTTAAAATACAAAGCTATCC	D39	60 nt of 3' *murMN* and 3' downstream fragment
P1538	GCCTCTGTCTTGGTATCATGACTTCCACGAA		
For construction of K270 (Δ*cps* Δ*pgdA*::P$_c$-*kan-rpsL*$^+$)			
P632	TGAAATGATGGCTGATAGCGCCAGTTCCGA	D39	5' upstream fragment with 60nt of 5' *pgdA*
P634	CATTATCCATTAAAAATCAAACGGATCCTATTTCCC GTGTCTGCCACGTCC		
Kan rpsL forward	TAGGATCCGTTTGATTTTTAATGGATAATG	P$_c$-*kan-rpsL*$^+$ cassette[b]	P$_c$-*kan-rpsL*$^+$ cassette
Kan rpsL reverse	GGGCCCCTTTCCTTATGCTTTTG		
P635	CAAAAGCATAAGGAAAGGGGCCCAGATGCTCAAT ACTCGCCTAAAAGCTC	D39	60 nt of 3' *pgdA* and 3' downstream fragment
P633	AACTCGTGGAGCACCCGATTGAACAGCAAT		
For construction of K447 (Δ*cps* Δ*adr*::P$_c$-*kan-rpsL*$^+$)			
P981	TCACCGTCGTCCACATGACCAAGGTTTGTT	D39	5' upstream fragment with 60nt of 5' *adr*
P983	CATTATCCATTAAAAATCAAACGGATCCTAATACAA GAGTACCAAAAGTAAACCTATA		
Kan rpsL forward	TAGGATCCGTTTGATTTTTAATGGATAATG	P$_c$-*kan-rpsL*$^+$ cassette[b]	P$_c$-*kan-rpsL*$^+$ cassette
Kan rpsL reverse	GGGCCCCTTTCCTTATGCTTTTG		
P984	CAAAAGCATAAGGAAAGGGGCCCGCAGATACGAT TGCCACAGCTTTG	D39	60 nt of 3' *adr* and 3' downstream fragment
P982	ACCTGTCGATTTGAGGCGTGCAGTTCCATT		

[a]Genomic DNA of the indicated *S. pneumoniae* strains was used as templates for PCR reactions, except for P$_c$-*erm* and P$_c$-*kan-rpsL*$^+$ cassettes.

[b]P$_c$-*erm* and P$_c$-*kan-rpsL*$^+$ cassettes are described in (5).

SUPPLEMENTAL FIGURE LEGENDS

FIG S1. Unlabeled cell poles of *S. pneumoniae* do not acquire new labeling. Cells of parent strain IU1945 (D39 Δ*cps*) were grown exponentially in BHI broth, labeled with HADA (pseudocolored green) for 30 min (A) or 105 min (B), washed, and chased in the presence of TADA (pseudocolored red) for 5 min as described in Materials and Methods. Live cells were imaged by epi-fluorescence phase-contrast microscopy, and arrows in (A) indicate blank hemispheres ("flattops") that were not labeled by HADA in 30 min and that are bordered by a red TADA band of new PG synthesis. Minimal variation in labeling patterns was observed for >300 individual cells in diplococci or short chains examined in microscopic fields, and representative images are shown. Scale bar = 1 μm. Fluorescence overlays of HADA and TADA labeling and phase overlays of HADA, TADA, and phase-contrast images are shown.

FIG S2. Similar unlabeled PG peptide profiles in parent strain IU1945 (D39 Δ*cps*) (A) and isogenic mutant IU7204 (D39 Δ*dacA*) (B), which lacks the D,D-carboxypeptidase that converts PG pentapeptides to PG tetrapeptides. Bacteria were grown in BHI broth without labeling (red lines) or with HADA labeling (blue lines), and PG was purified and PG peptides released with purified LytA amidase as described in Materials and Methods. HPLC was used to resolve PG peptides using the gradient conditions described in (6) with UV absorbance detection at 210 nm (A_{210}). Different chromatograms are offset on the same x-time axis for comparisons of unlabeled and labeled samples. Arrows indicate peaks of HADA-labeled PG peptides not present in unlabeled samples. The experiment was performed independently twice with similar

results. Note that the HPLC gradient here was different from that used in Figure 4 (see Materials and Methods; (4)). See text for additional details.

FIG. S3. Persistence of hemispheres of stable old PG indicative of minimal turnover in cells of *S. pneumoniae* TIGR4 and D39 encapsulated strains visualized by long-pulse/chase/new-labeling with FDAA probes. IU1686 (TIGR4) and IU1690 (D39) (see Table S1) were grown exponentially in BHI broth, labeled with HADA (pseudocolored green; old PG), washed, and chased in the presence of either NADA or TADA (pseudocolored red; newly synthesized PG) as described for Figure 2 and in Materials and Methods. Images are representative of >200 individual cells in diplococci or chains of each strain examined in microscopic fields at different time points after the start of the chase/second FDAA labeling in two biological replicates. Scale bar = 1 μm. P, phase contrast image; H, HADA labeling (old PG); T, TADA or N, NADA labeling (new PG synthesis); O, overlay of H and T or N images. Note that encapsulated strain D39 forms short chains of cells compared to unencapsulated mutants (Fig. 2) as we reported previously (3).

FIG. S4. Persistence of hemispheres of stable old PG indicative of minimal turnover in wild-type (parent) bacteria growing in chemically defined medium (CDM) containing glucose or galactose as carbon source. Strain IU1945 (D39 Δ*cps*) was grown exponentially in CDM medium with the indicated carbon sources, labeled with HADA (pseudocolored green; old PG), washed, and chased in the presence of NADA (pseudocolored red; newly synthesized PG) as described for Figure 2 and in Materials and Methods. Images are representative of >124 individual cells in diplococci or short chains for each growth condition examined in microscope fields at different time points

after the start of the chase/NADA labeling in two biological replicates. Scale bar = 1 μm. P, phase contrast image; H, HADA labeling (old PG); N, NADA labeling (new PG synthesis); O, overlay of H and images.

Fig. S5. Persistence of hemispheres of stable old PG indicative of minimal turnover turnover in laboratory strain R6 or a D39 Δ*cps* Δ*murMN* mutant with reduced or no PG cross-bridges, respectively, compared to the D39 Δ*cps* parent strain. Cells of EL59 (R6) and IU9709 (D39 Δ*cps* Δ*murMN*) (see Table S1) were grown exponentially in BHI broth, labeled with HADA (pseudocolored green; old PG), washed, and chased in the presence of TADA (pseudocolored red; newly synthesized PG) as described for Figure 2 and in Materials and Methods. Images are representative of >200 individual cells in diplococci or short chains of each strain examined in microscopic fields at different time points after the start of the chase/NADA labeling in two biological replicates. Scale bar = 1 μm. P, phase contrast image; H, HADA labeling (old PG); T, TADA labeling (new PG synthesis); O, overlay of H and T images. Note that IU9709 and IU1945 (Fig. 2) are isogenic (see Table S1).

Fig. S6. Full time courses of long-pulse/chase/new-labeling experiments of the Δ*pbp1a* and PG hydrolase mutants shown in Figure 8. Strains and labeling are described in the legend to Figure 8, which shows only two time-points for each strain. Old PG labeled with HADA is pseudocolored green, whereas regions of new PG synthesis are pseudocolored red. At least two biological replicates were done for each strain. The number of individual cells examined for each strain is indicated. Scale bar = 1 μm. P, phase contrast image; H, HADA labeling (old PG); N, NADA labeling (new PG synthesis); O, overlay H and N images.

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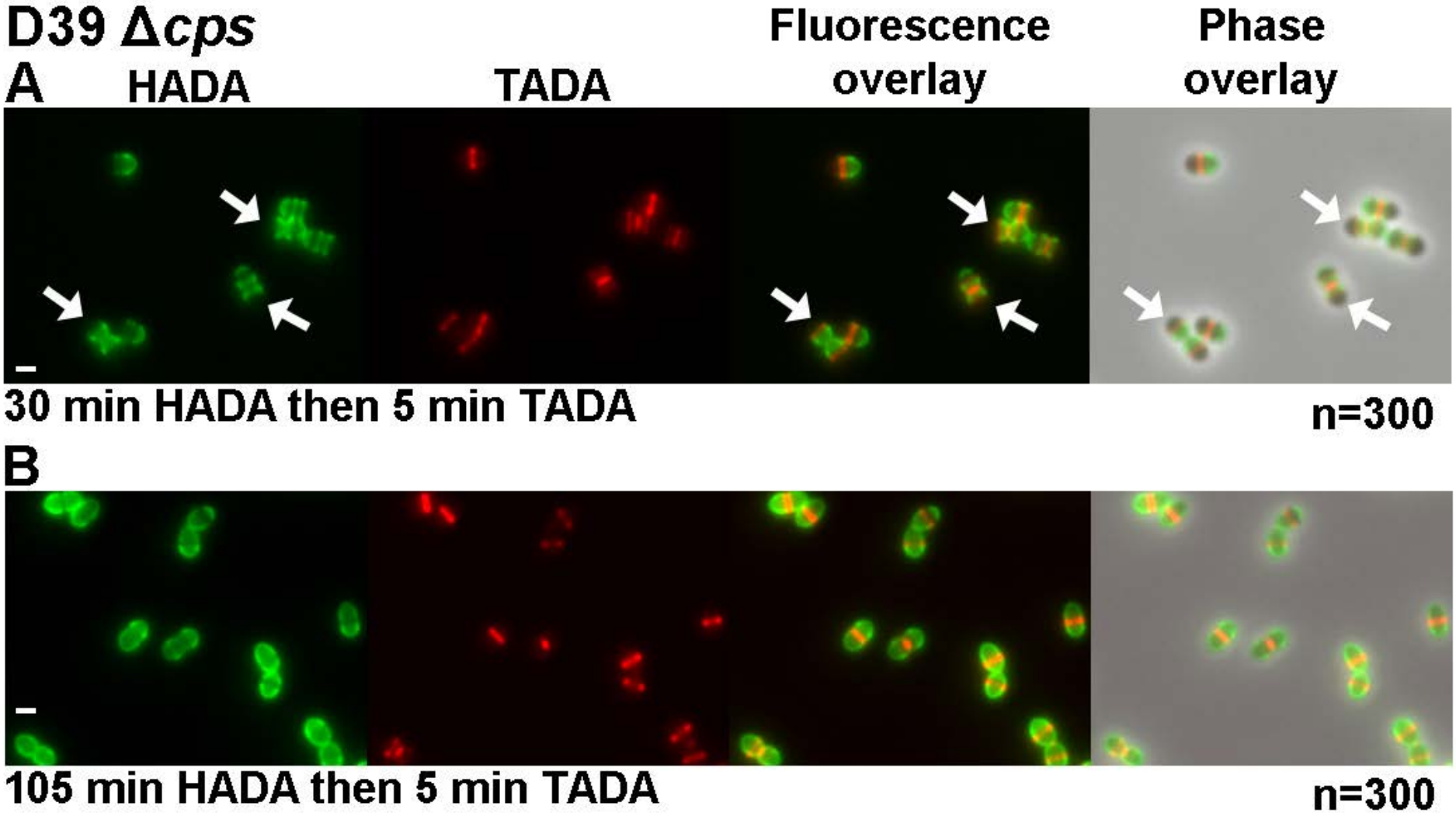
D39 Δcps
A
HADA
TADA
Fluorescence overlay
Phase overlay
30 min HADA then 5 min TADA
n=300
B
105 min HADA then 5 min TADA
n=300

Fig. S1

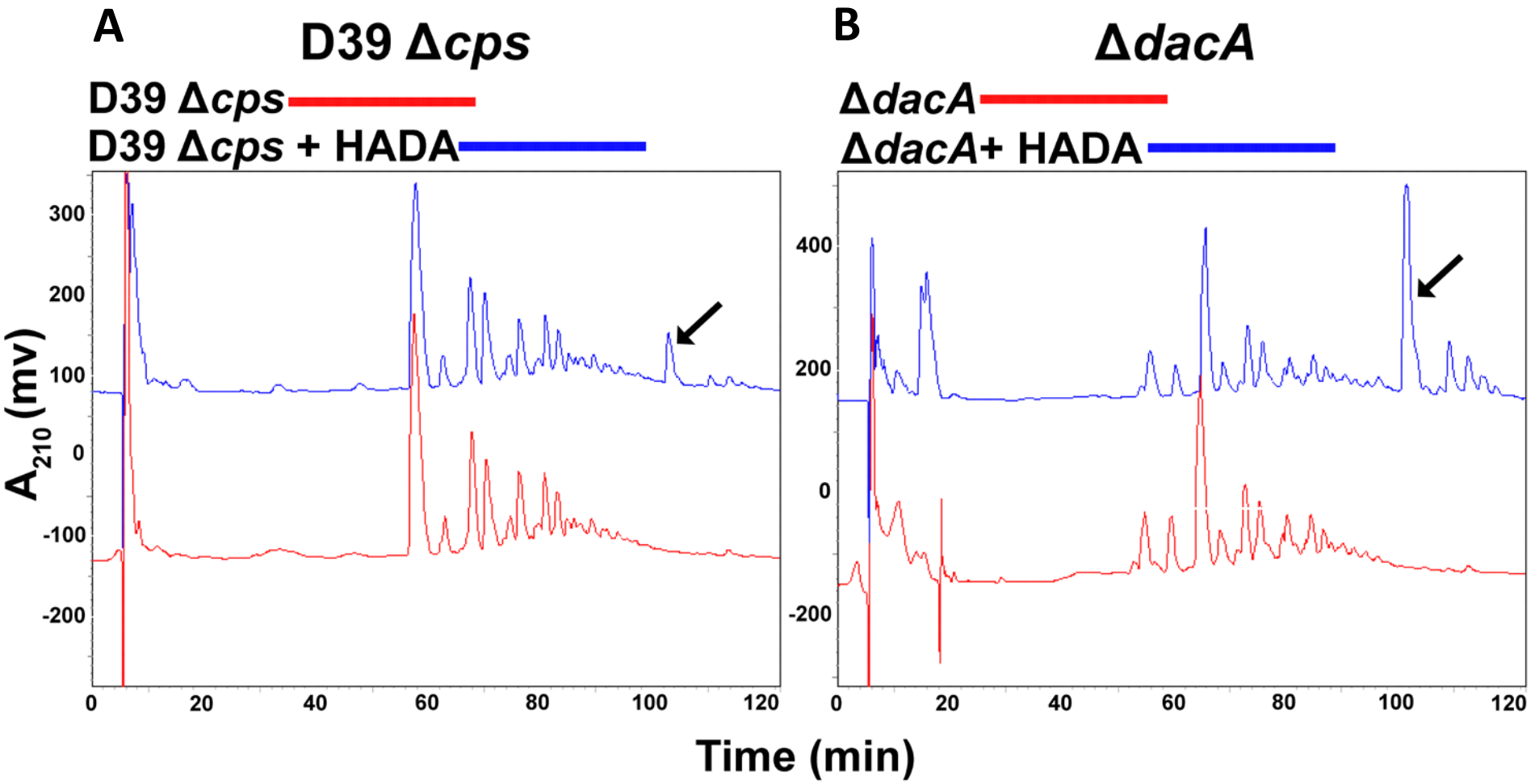
A
D39 Δcps
D39 Δcps
D39 Δcps + HADA
B
ΔdacA
ΔdacA
ΔdacA+ HADA
A210 (mv)
300
200
100
0
-100
-200
400
200
0
-200
0
20
40
60
80
100
120
Time (min)

Fig. S2

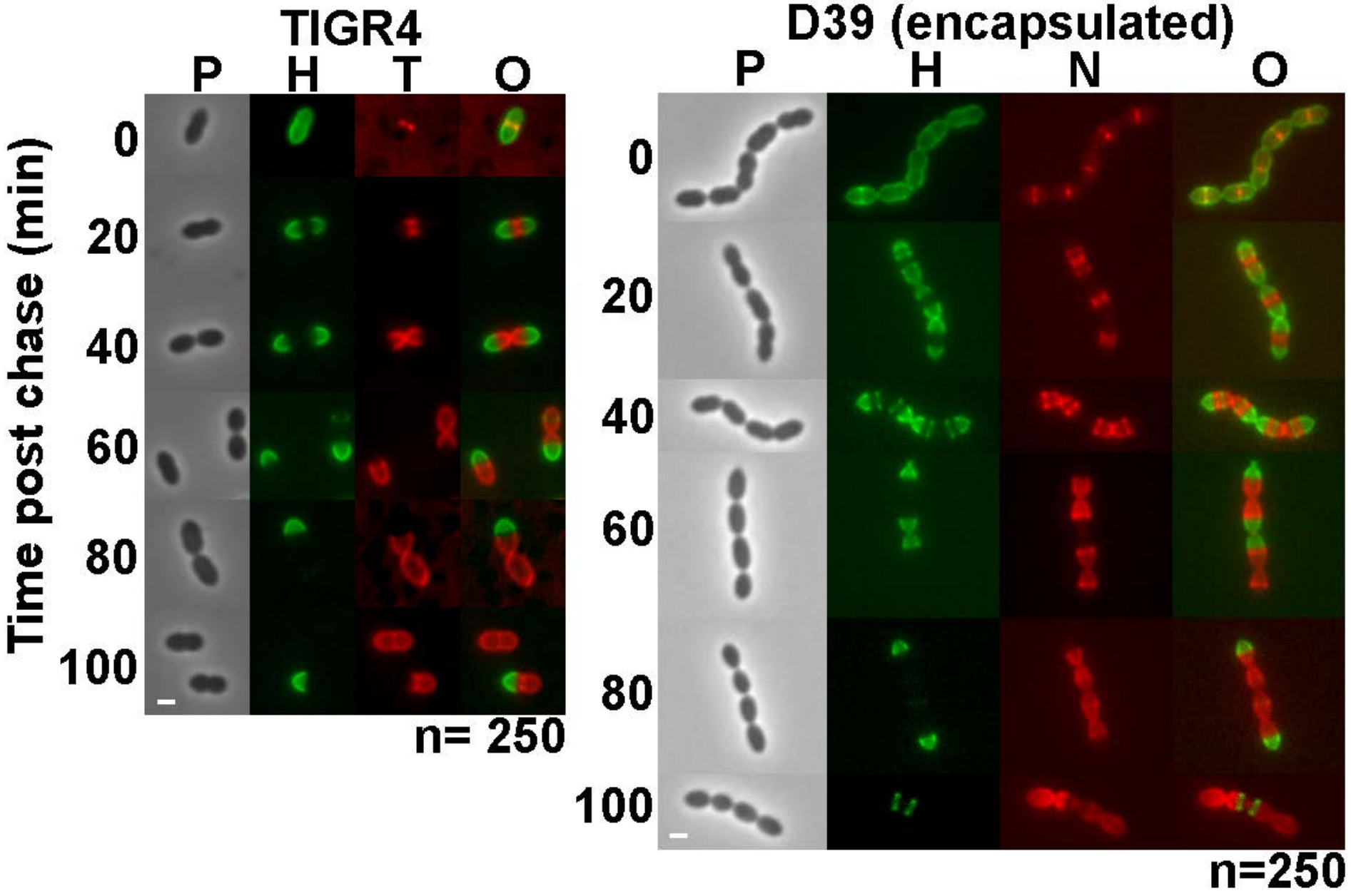
TIGR4
P
H
T
O
D39 (encapsulated)
P
H
N
O
Time post chase (min)
0
20
40
60
80
100
n= 250
n=250

Fig. S3

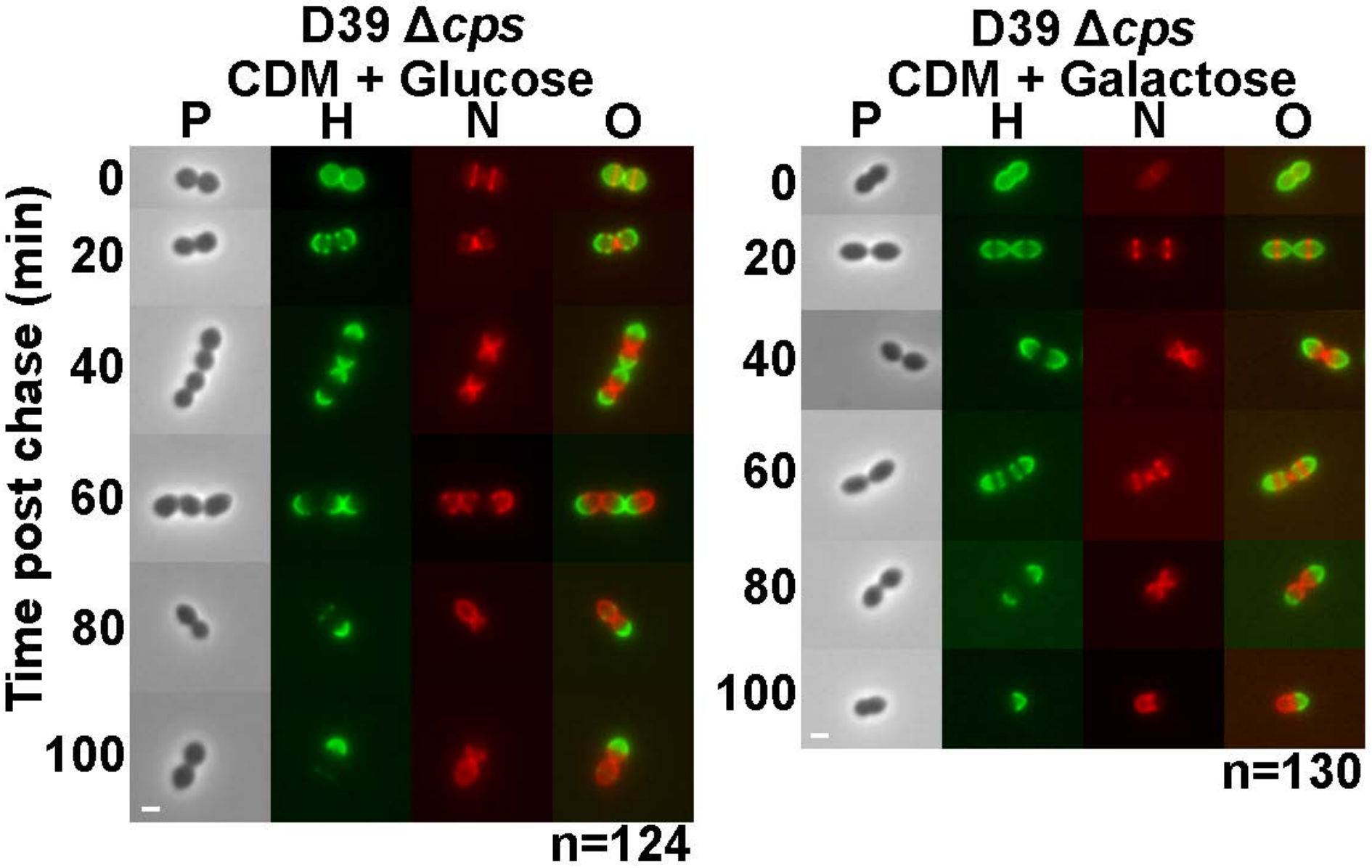
D39 Δcps
CDM + Glucose
P
H
N
O
Time post chase (min)
0
20
40
60
80
100
n=124
D39 Δcps
CDM + Galactose
P
H
N
O
0
20
40
60
80
100
n=130

Fig. S4

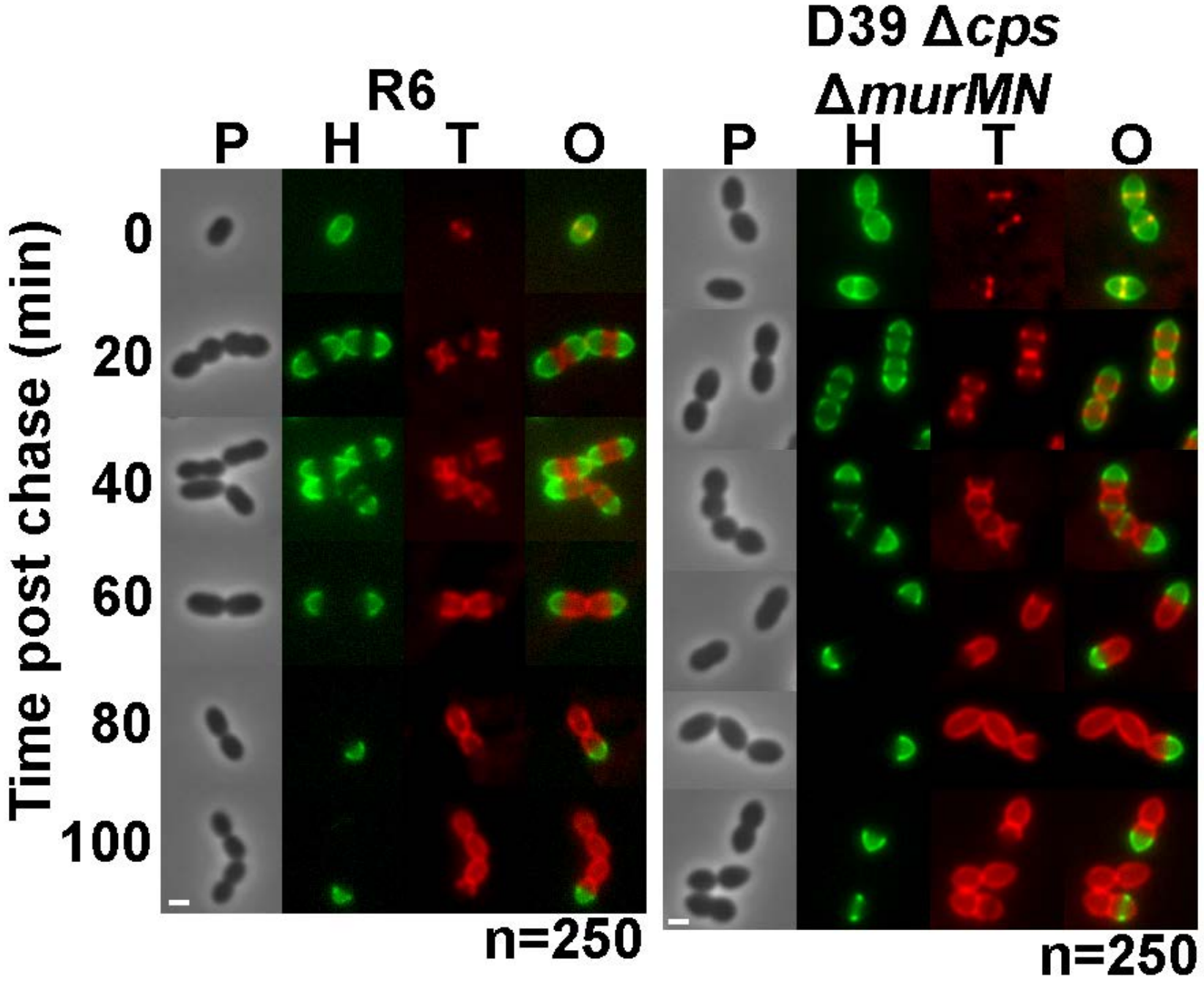
R6
D39 Δcps ΔmurMN
P
H
T
O
P
H
T
O
Time post chase (min)
0
20
40
60
80
100
n=250
n=250

Fig. S5

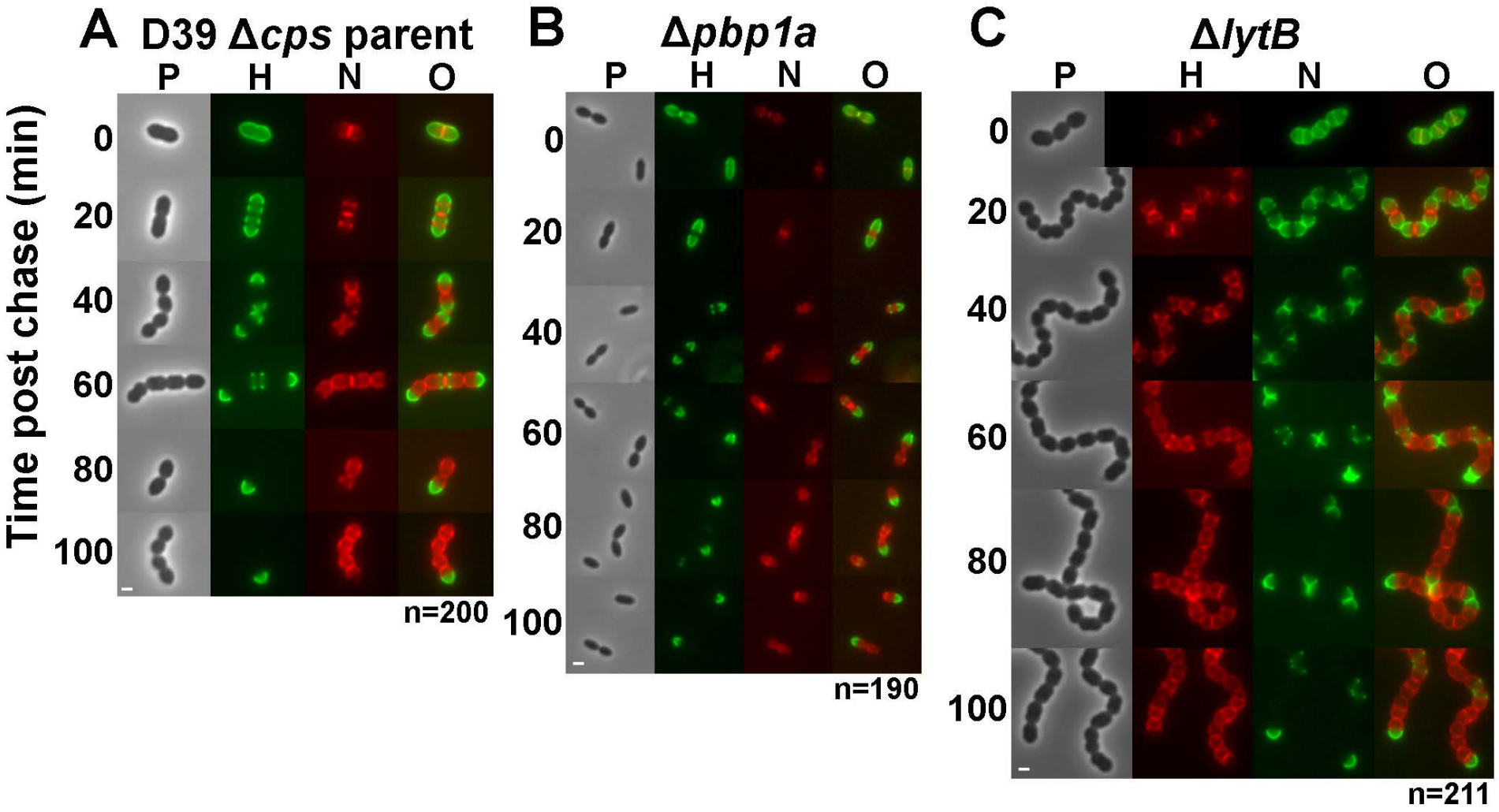

Fig. S6 (continued on next pages)

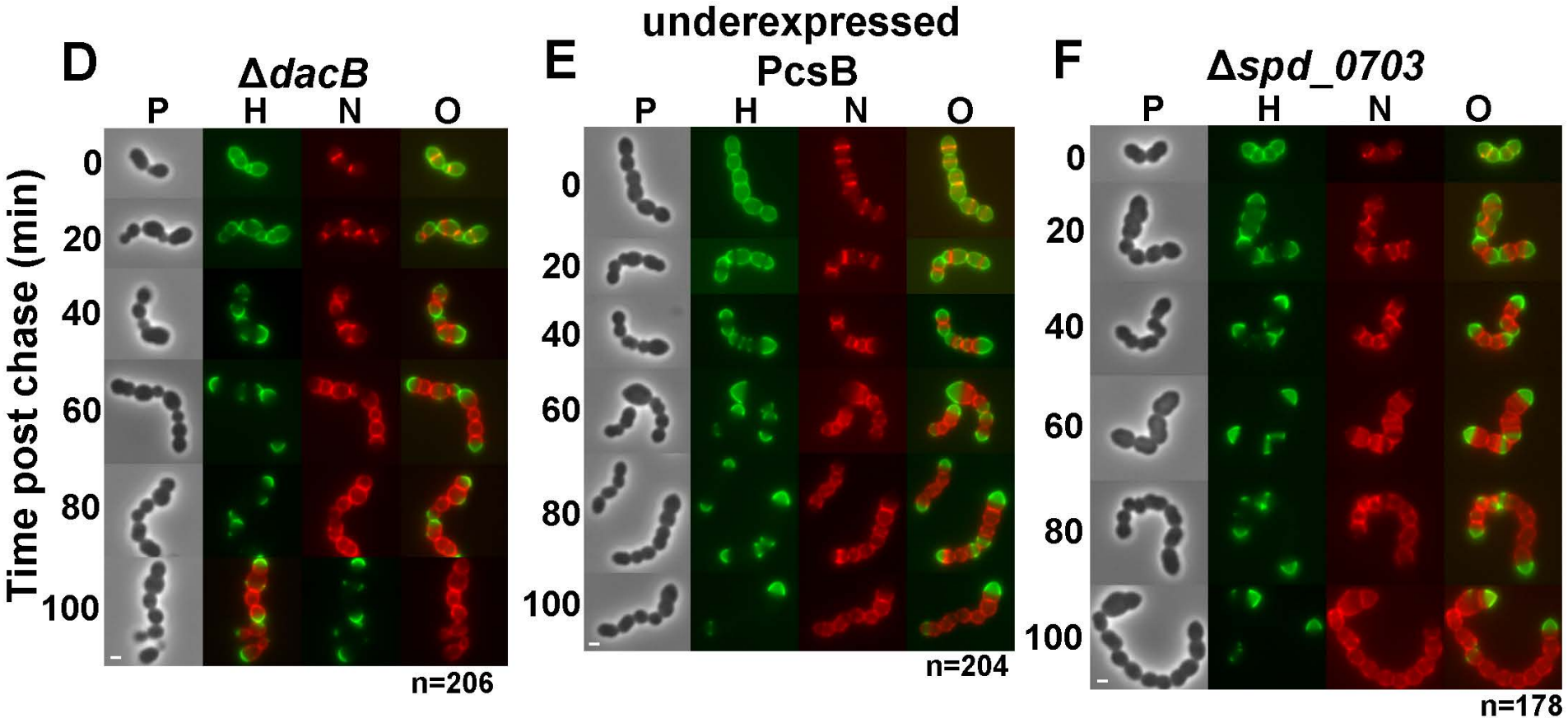

Fig. S6 (continued on next pages)

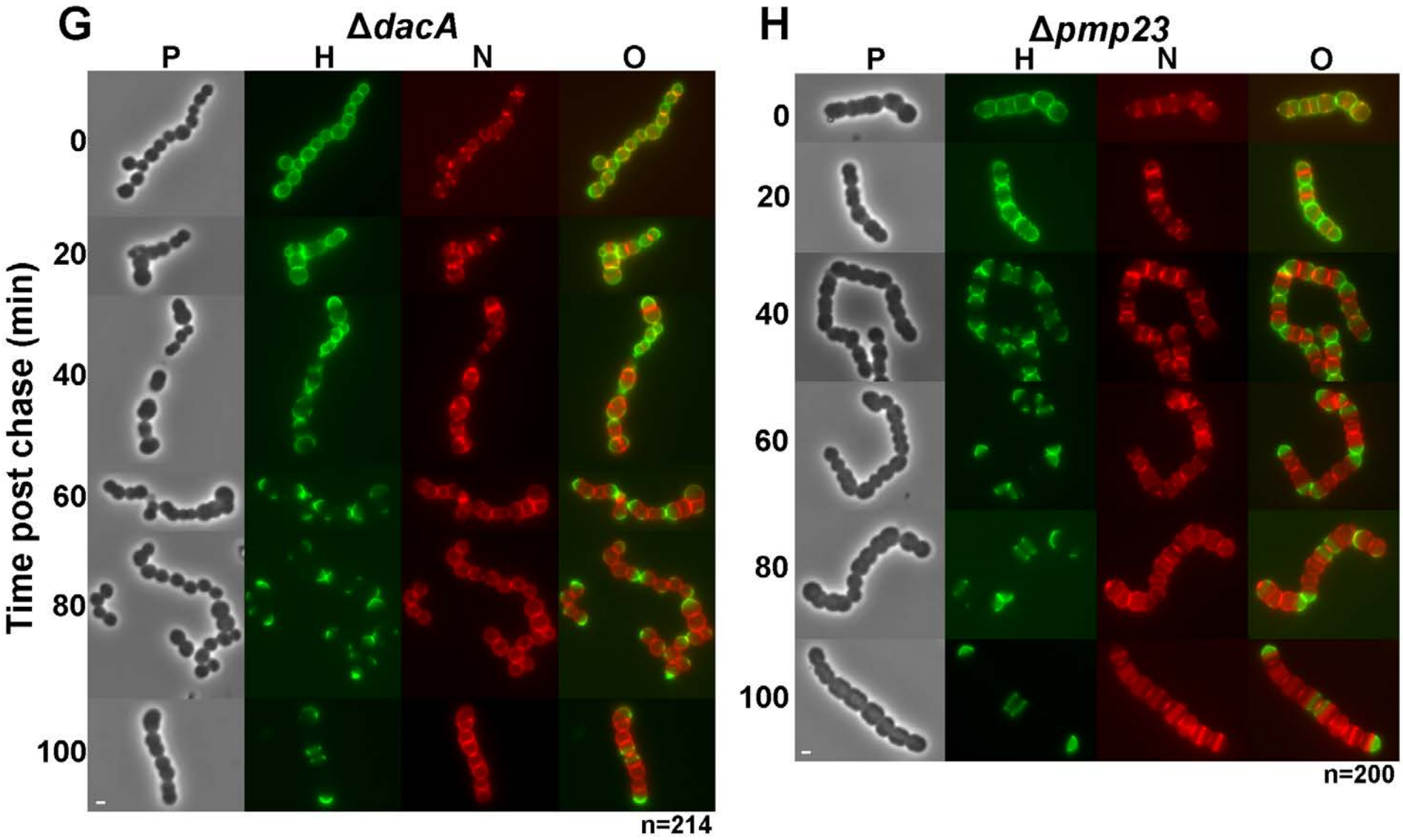

Fig. S6 (continued)