Supporting Information

Table S1, Specific activities of enzyme preparations

E. coli	
pIY003 (WT)	
Evolution specific activity	0.91±0.10 nmH_2 min^{-1} (mg lysate)$^{-1}$
Uptake specific activity	4.8±0.6 nmH_2 min^{-1} (mg lysate)$^{-1}$
Relative activity after 85 °C, 1h	41% (std. err. 39-45%)*
Relative activity in 1% O_2	29% (std. err. 21-35%)*
pIY004 (H230C)	
Evolution specific activity	0.50±0.05 nmH_2 min^{-1} (mg lysate)$^{-1}$
Uptake specific activity	9.2±2.9 nmH_2 min^{-1} (mg lysate)$^{-1}$
pIY006(P285C)	
Evolution specific activity	0.35±0.04 nmH_2 min^{-1} (mg lysate)$^{-1}$
Uptake specific activity	7.7±1.2 nmH_2 min^{-1} (mg lysate)$^{-1}$
pIY007(H230C/P285C)	
Evolution specific activity	3.3±0.4 nmH_2 min^{-1} (mg lysate)$^{-1}$
Uptake specific activity	5.9±2.4 nmH_2 min^{-1} (mg lysate)$^{-1}$
Relative activity after 85 °C, 1h	27% (std. err. 17-35%)*
Relative activity in ~1% O_2	37% (std. err. 23-48%)*
Specific activity relative to pIY003	3.7±0.6
pIY033(WT)	
Evolution specific activity	1.6±0.3 nmH_2 min^{-1} (mg lysate)$^{-1}$
pIY038(H230C/P285C)	
Evolution specific activity	3.8±0.5 nmH_2 min^{-1} (mg lysate)$^{-1}$
Specific activity relative to pIY033	2.3±0.8
pRC41-4(WT)	
Evolution specific activity	9.1 nmH_2 min^{-1} (mg lysate)$^{-1}$
pCM012(H230C/P285C)	
Evolution specific activity	19 nmH_2 min^{-1} (mg lysate)$^{-1}$
pIY085(WT, strep-tagged)	
Evolution specific activity, unpurified	6.1 nmH_2 min^{-1} (mg lysate)$^{-1}$
Evolution specific activity, purified	690 nmH_2 min^{-1} (mg protein)$^{-1}$
pIY086(H230C/P285C, strep-tagged)	
Evolution specific activity, unpurified	12.7 nmH_2 min^{-1} (mg lysate)$^{-1}$
Evolution specific activity, purified	2200 nmH_2 min^{-1} (mg protein)$^{-1}$
Purified specific activity relative to pIY085	3.2
S. elongatus	
pRC41-4(WT)	
Evolution specific activity	0.10±0.01 nmH_2 min^{-1} (mg lysate)$^{-1}$
pCM012(H230C/P285C)	
Evolution specific activity	0.54±.06 nmH_2 min^{-1} (mg lysate)$^{-1}$
Specific activity relative to pRC41-4	5.1±0.7

*geometric standard error

Table S2, Primers used in this study

Name	Sequence	Purpose
AMR3-2	**CAATTTCACACAGGAAACAGACC**ATGGATG GCTTCCATTATTGGTAGC	Forward primer for amplification of hydrogenase operon starting at orf2 (light); overlaps with pTRC-NS1 (bold)
AMR-44	**TGCATGCCTGCAGGTCGACTCTAGAGGATC** TCAGCAGATCCTTGGAAGC	Reverse primer for amplification of hydrogenase operon ending at hypE (light); overlaps with pTRC-NS1(bold)
IY002HopMidF	CCAGGCTACTCGAAGAAACG	Forward primer in the middle of hydrogenase operon
IY003HopMidR	GCGCACGTATCGTTTCTTCG	Reverse primer in the middle of the hydrogenase operon
IY004HydSH238CF	TGACAGATGTTATCGACGCCC	Forward primer to generate H230C
IY005HydSH238CR	GGGCGTCGATAACATCTGTC**ACA**GATGCTT TCACCAAAAAAAG	Reverse primer to generate H230C mutation (bold)
IY010pDEST24F	GGCTGCTGCCACCGCTGAGC	Forward primer to amplify pDEST backbone
IY011pDEST24R	CTATAGTGAGTCGTATTAATTTCGCGGG	Reverse primer to amplify pDEST backbone
IY012HydT7CBAF	**CCCGCGAAATTAATACGACTCACTATAG**GG AAACAGACCATGGATGGC	Forward primer to amplify hydrogenase operon for insertion into pDEST24; overlaps with IY011pDEST24R (bold)
IY013HydT7CBAR	**GCTCAGCGGTGGCAGCAGCC**AGGATCTCAG CAGATCCTTGG	Reverse primer to amplify hydrogenase operon for insertion into pDEST24; overlaps with IY010pDEST24F (bold)
IY014pDEST24F	CCCGCGAAATTAATACGACTCACTATAG	Forward primer to amplify hydrogenase operon out of pDEST24 plasmid
IY015pDEST24R	CTCGGAGCACTGTCCGACCGC	Reverse primer to amplify hydrogenase operon out of pDEST24 plasmid
IY016NS1pDEST24F	**GCGGTCGGACAGTGCTCCGAG**CGAGCTTCT GGAGCAGGAAG	Forward primer to amplify pTRC-NS1 for insertion of T7-driven hydrogenase; overlaps with IY015pDEST24R(bold)
IY017NS1pDEST24R	**CTATAGTGAGTCGTATTAATTTCGCGGG**CC GATCCTCTAGTATGCTTGTAAACC	Reverse primer to amplify pTRC-NS1 for insertion of T7-driven hydrogenase; overlaps with IY014pDEST24F(bold)
IY018HydSP293CF	ATCGAATCTGGCCATCCGTGT	Forward primer to generate P285C
IY019HydSP293CR	ACACGGATGGCCAGATTCGAT**ACA**AAAACT CGTGCCTTG	Reverse primer to generate P285C mutation (bold)
IY170HynSF	GTATTATTGCCATCGGTACC	Forward primer upstream of BamHI site to transfer mutants
IY171HynSR	CGAGTGATAGGATCGACGAC	Reverse primer downstream of AgeI site to transfer mutants
AN000StrepF	CGTAGCGCTTGGTCCCACCCGCAGTTCGAA AAA**TAAGATAATAGGCAAGGCTCATGG**	Forward primer encoding the HynS C-terminal strep-tag; overlaps with hynS-hynL intergenic region (bold)
AN001StrepR	TTTTTCGAACTGCGGGTGGGACCAAGCGCT ACGTGAG**TTTTTCCCAGCATGCTTC**	Reverse primer encoding the HynS C-terminal strep-tag; overlaps with the c-terminus of hynS (bold)

Table S3 (22pp), Maps and sequences for vectors used in this study

Map Key:

Green – replication origins, transfer origins, transcriptional promoters

oriV – vegetative origin from p15

oriT – RP4 conjugation transfer origin

PTRC – P_{TRC} promoter (includes lacO repressor element)

PT7 – P_{T7} promoter

lacO – lacO repressor element

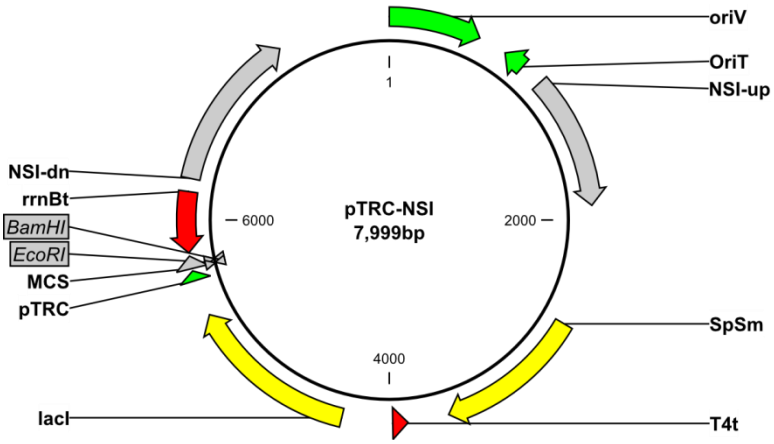
Red – transcriptional terminators, mutations

T4t – T4 terminator

rrnBt – rrnB terminator

Grey – upstream and downstreamNS1 homology regions for transfer into *S. elongatus*

Yellow – ORFs



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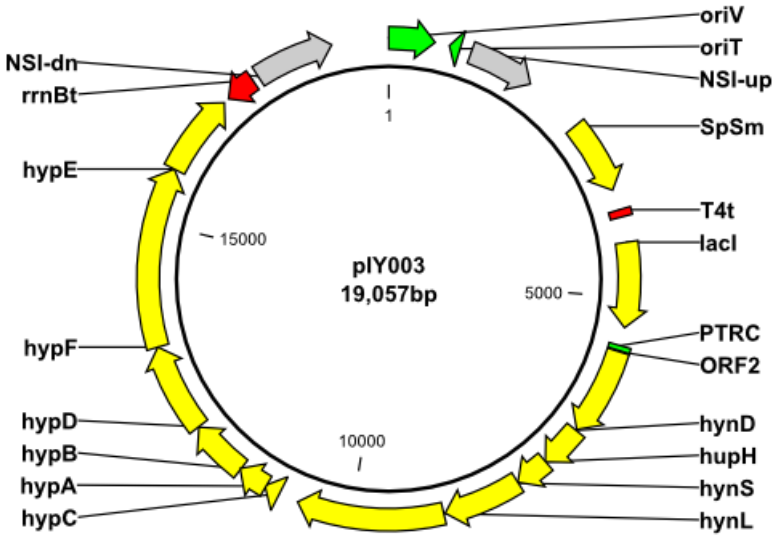

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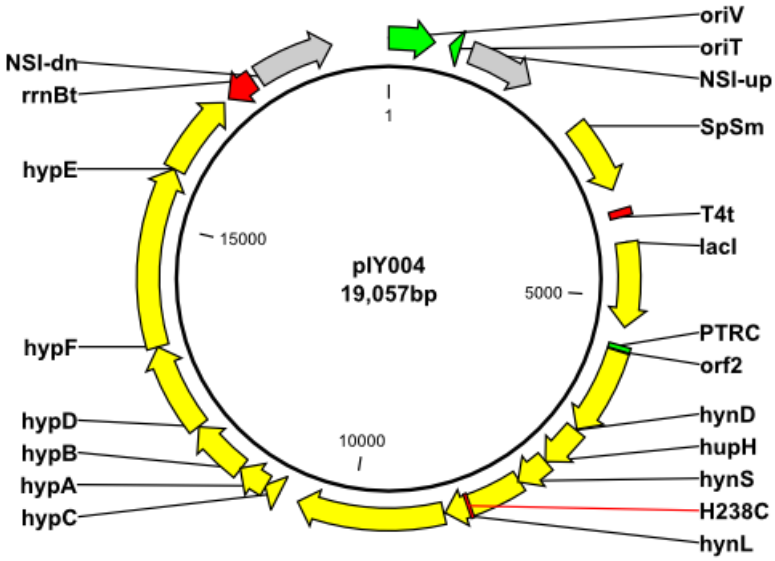

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GGGTCCCCAATTAATTAGTAATATAATCTATTAAAGGTCATTCAAAAGGTCATCCACCGGATCAATTCCCCTGCTCGCGCAGGCTGGGTGCCAAGCTCTCGGGTAACATCAAGGCCCGATCCTTGGAGCCCTTGCCCTCCCGCACGATGATCGTGCCGTGATCGAAATCC
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AAGCCAGGGCAGATCCGTGCACAGCACCTTGCCGTAGAAGAACAGCAAGGCCGCCAATGCCTGACGATGCGTGGAGACCGAAACCTTGCGCTCGTTCGCCAGCCAGGACAGAAATGCCTCGACTTCGCTGCTGCCCAAGGTTGCCGGGTGACGCACACCGTGGAAACGGA
TGAAGGCACGAACCCAGTGGACATAAGCCTGTTCGGTTCGTAAGCTGTAATGCAAGTAGCGTATGCGCTCACGCAACTGGTCCAGAACCTTGACCGAACGCAGCGGTGGTAACGGCGCAGTGGCGGTTTTCATGGCTTGTTATGACTGTTTTTTTGGGGTACAGTCTATG
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TGGAGAGAGCGAGATTCTCCGCGCTGTAGAAGTCACCATTGTTGTGCACGACGACATCATTCCGTGGCGTTATCCAGCTAAGCGCGAACTGCAATTTGGAGAATGGCAGCGCAATGACATTCTTGCAGGTATCTTCGAGCCAGCCACGATCGACATTGATCTGGCTATCT
TGCTGACAAAAGCAAGAGAACATAGCGTTGCCTTGGTAGGTCCAGCGGCGGAGGAACTCTTTGATCCGGTTCCTGAACAGGATCTATTTGAGGCGCTAAATGAAACCTTAACGCTATGGAACTCGCCGCCCGACTGGGCTGGCGATGAGCGAAATGTAGTGCTTACGTTG
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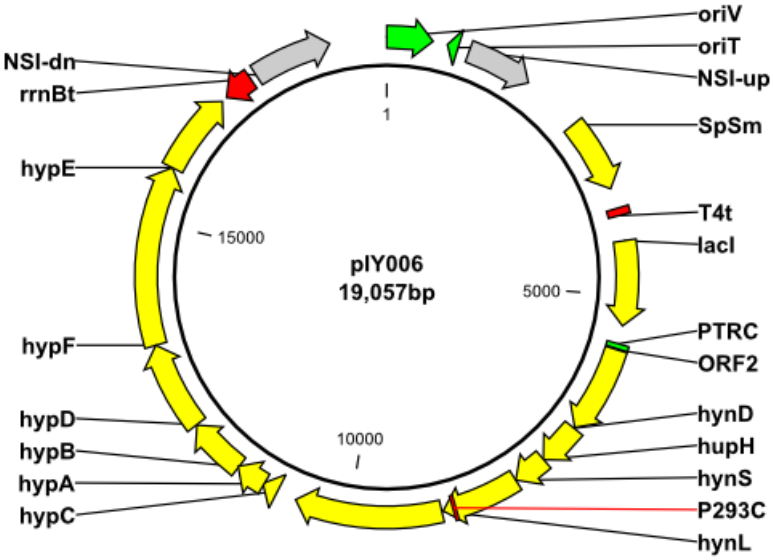

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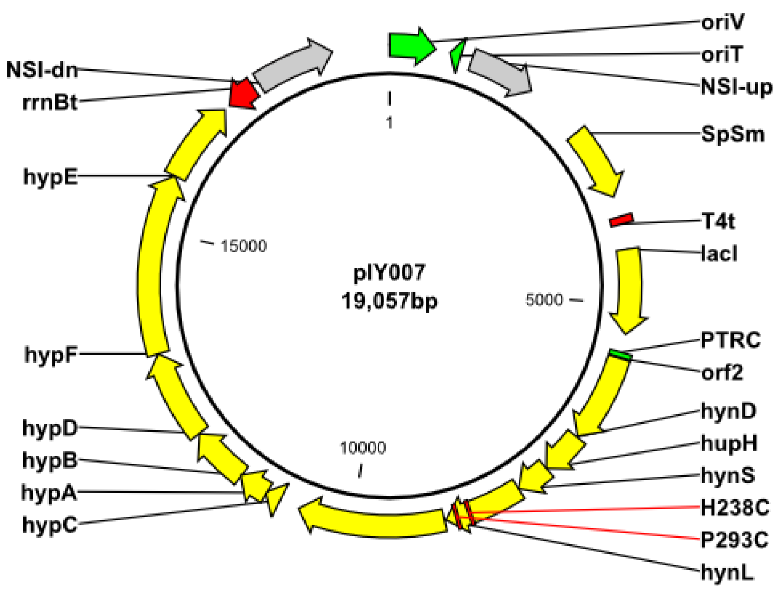

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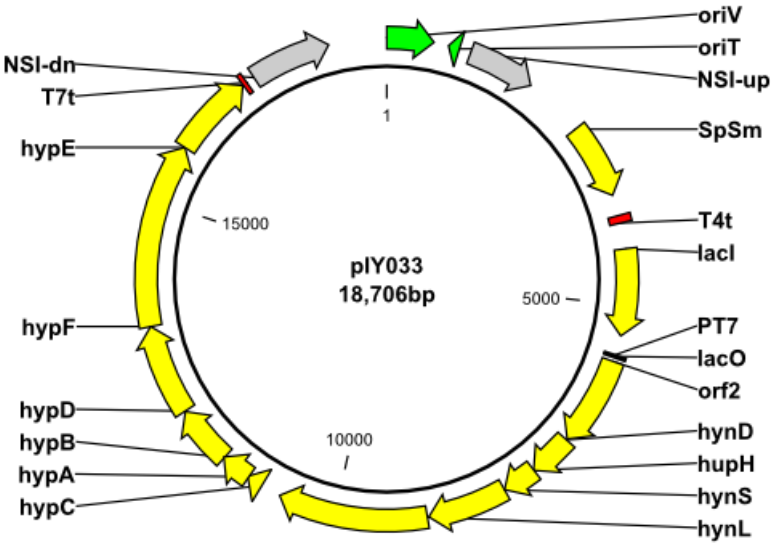

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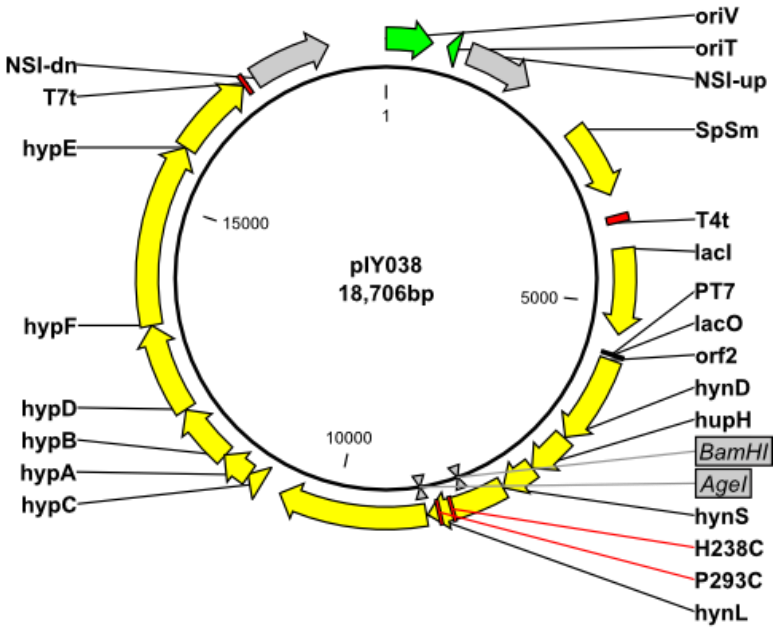

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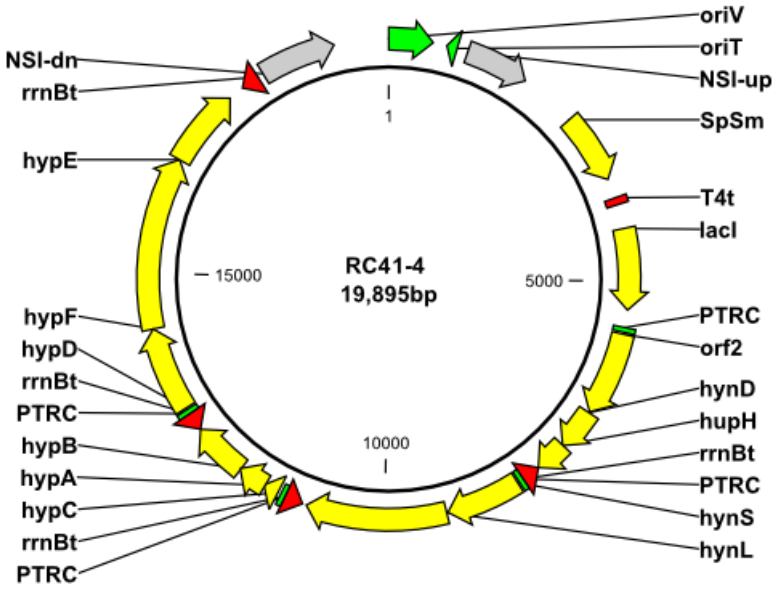

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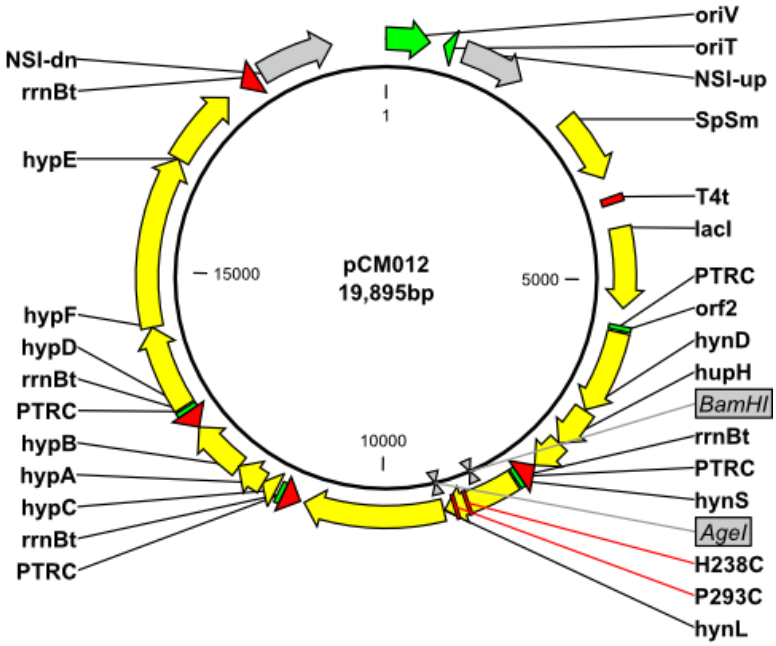

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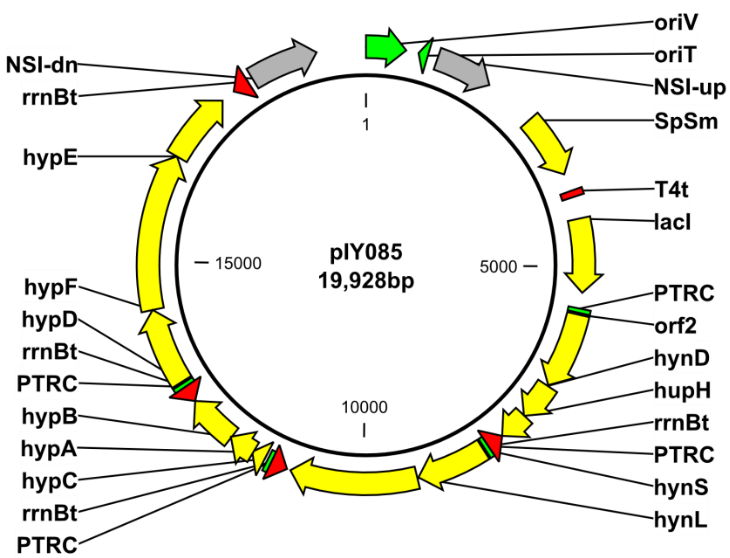

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TGGAGAGAGCGAGATTCTCCGCGCTGTAGAAGTCACCATTGTTGTGCACGACGACATCATTCCGTGGCGTTATCCAGCTAAGCGCGAACTGCAATTTGGAGAATGGCAGCGCAATGACATTCTTGCAGGTATCTTCGAGCCAGCCACGATCGACATTGATCTGGCTATCT
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CGACTGCACGGTGCACCAATGCTTCTGGCGTCAGGCAGCCATCGGAAGCTGTGGTATGGCTGTGCAGGTCGTAAATCACTGCATAATTCGTGTCGCTCAAGGCGCACTCCCGTTCTGGATAATGTTTTTTGCGCCGACATCATAACGGTTCTGGCAAATATTCTGAAATG
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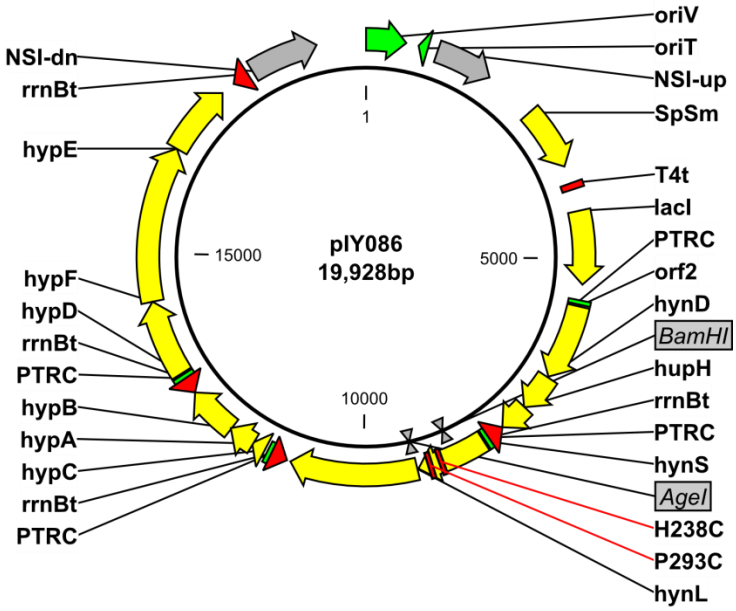

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TGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTC

Figure S1, A representative enzyme activity progress curve over four hours, using the doubly substituted enzyme expressed in BL21ΔH_4 cells. Data points represent three technical replicates of an experiment conducted using the procedures described in the methods section. There is some deviation from linearity at 12h, likely due to consumption of reductive potential.

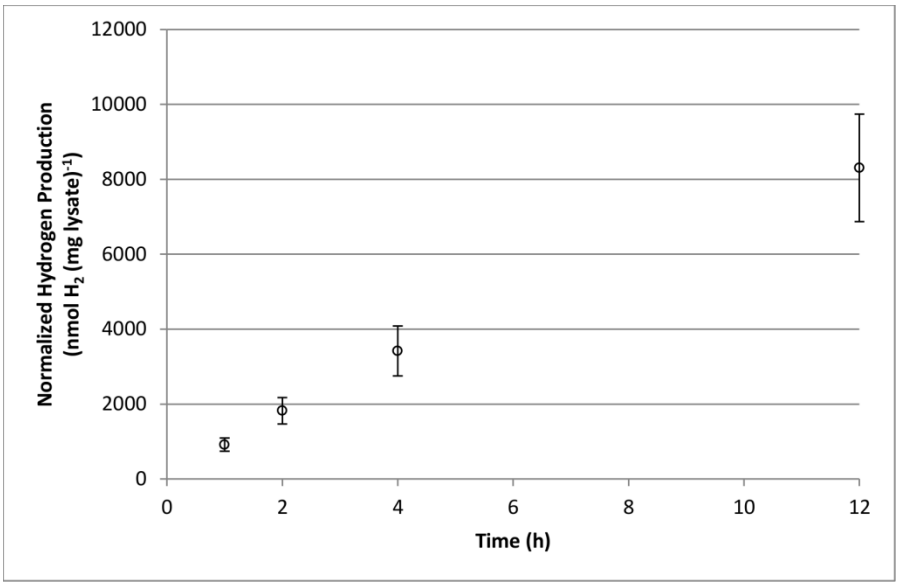

Figure S2, Specific activities of samples from western blot experiment (bars correspond to samples from gel lanes as found in main text Fig. 4)