Supplemental Information

Methods

Strain construction

Mutations in *trnD-Phe*, *hrcA*, *tyrS*, and *ppaC* were markerlessly reconstructed in *B. subtilis* 3610 using the pMiniMAD protocol as described (1) and primers 39-42 and 45-50. Successful double-crossover events were indicated by the absence of growth in the presence of erythromycin and lincomycin. Mutations were subsequently verified using primers 45, 46, 49, 50, and 57-60.

Luciferase reporters for readthrough of the *tyrS* riboswitch were constructed as follows. pAH328 (2) was digested with EcoRI and BamHI and a fragment of approximately 6 kb, containing the optimized, promoterless *luxABCDE* operon, was isolated by gel extraction. The *amyE* integrating vector pDG1662 (3) was digested with EcoRI and BamHI and a fragment of approximately 7 kb was isolated by gel extraction. The fragments were ligated and transformed into *E. coli* DH5α, generating pLF007, and the construct was confirmed by restriction digestion using HindIII and PstI. The promoter region of *tyrS* (4) was amplified from *B. subtilis* 3610, SLH8, and SLH9 using primers 70 and 71, or from 3610 using primers 70 and 72, and combined by isothermal assembly (14) with pLF007 linearized with NotI and EcoRI to yield pSL001, pSL002, pSL003, and pSL008, respectively. The plasmids were maintained in *E. coli* Turbo in the presence of ampicillin, the promoter region sequences were verified with primers LF002F and LF002R, and the plasmids were transformed into *B. subtilis* 168. The constructs were moved by phage transduction into 3610 or SLH36 to yield strains SLH57, SLH58, SLH59, SLH61, and SLH75. Movement of the plasmids into *B. subtilis* was confirmed by growth on LB

agar containing chloramphenicol and proper insertion at *amyE* was verified by the inability of the strains to metabolize starch.

To construct the wild-type *tapA* complementation strain, primers AE1 and AE2 were used to amplify a fragment containing the *tapA* operon, including 200 bp upstream of the operon representing the promoter, from *B. subtilis* 3610 chromosomal DNA. To generate the mutated *tapA* variants, we created two fragments of the *tapA* operon overlapping at the desired mutation site, including the promoter, using primers AE1-AE6. The corresponding fragments were then joined in an isothermal assembly reaction (5) with pDG364 (6) linearized with BamHI and EcoRI to produce pAE164 (wild-type *tapA*), pAE165 ($tapA^{558\Delta}$), or pAE166 ($tapA^{724\Omega G}$). The plasmids were maintained in *E. coli* DH5α using selection with ampicillin. After verification of the *tapA* sequences with primers 55 and 56, the plasmids were transformed directly into SLH63, a derivative of *B. subtilis* 3610 lacking the *tapA* operon at the native locus. SLH63 was generated by phage transduction (7) of *tapA-sipW-tasA*::*spec* from strain RL4602. Movement of the plasmids into *B. subtilis* was confirmed by growth on LB agar containing chloramphenicol and proper insertion at *amyE* was verified by the inability of the strains to metabolize starch.

The *tyrS* overexpression strains were constructed as follows. Primers 76 and 77 were used to amplify the fragment containing the *tyrS* riboswitch, ribosome binding site (RBS), and open reading frame (ORF) from *B. subtilis* 3610 chromosomal DNA. Primers 76 and 86 were used to amplify the fragment containing the *tyrS* RBS and ORF from *B. subtilis* 3610 chromosomal DNA. The resulting fragments were then joined in an isothermal assembly reaction (5) with pDR111 (8) linearized with HindIII and SphI to produce pSL004 (*tyrS* riboswitch, RBS, and ORF) or pSL005 (*tyrS* RBS and ORF). The plasmids were maintained in *E. coli* Turbo using selection with ampicillin. After verification of the plasmid inserts with

primers pDR111F and pDR111R, the plasmids were transformed directly into *B. subtilis* 3610 to generate strains SLH69 and SLH70. Movement of the plasmids into *B. subtilis* was confirmed by growth on LB agar containing spectinomycin and proper insertion at *amyE* was verified by the inability of the strains to metabolize starch.

Table S1. Strains, plasmids, and primers used in this study.

Strain	Genotype or description	Source
NCIB3610	Parental strain (wild-strain) *B. subtilis*	Laboratory stock
168	Lab strain of *B. subtilis, trpC2*	Laboratory stock
SLH7	Spontaneous D-LMWY resistance mutant, *ppaC*$^{434C>T}$	This study
SLH8	Spontaneous D-LMWY resistance mutant, *tyrS*$^{-38\Omega C}$	This study
SLH9	Spontaneous D-LMWY resistance mutant, *tyrS*$^{-38C>T}$	This study
SLH10	Spontaneous D-LMWY resistance mutant, *ppaC*$^{234A>T}$	This study
SLH13	Spontaneous D-LMWY resistance mutant, *trnD-Phe*$^{35A>T}$	This study
SLH15	Spontaneous D-LMWY resistance mutant, *hrcA*$^{-8A>G}$ *ppaC*$^{\Delta 496-498}$	This study
SLH16	Spontaneous D-LMWY resistance mutant, *tyrS*$^{605G>A}$	This study
SLH31	*dtd*$^{2T>A}$ in NCIB3610	(9)
ALM89	*sacA::P*$_{epsA}$*-lux* in NCIB3610, Cm^{R}	(10)
SLH32	*sacA::P*$_{epsA}$*-lux* in SLH31, Cm^{R}	This study
ALM91	*sacA::P*$_{tapA}$*-lux* in NCIB3610, Cm^{R}	(10)
SLH33	*sacA::P*$_{tapA}$*-lux* in SLH31, Cm^{R}	This study
SLH34	*ppaC*$^{434C>T}$ in NCIB3610	This study
SLH35	*tyrS*$^{-38C>T}$ in NCIB3610	This study
SLH36	*trnD-Phe*$^{35A>T}$ in NCIB3610	This study
SLH37	*hrcA*$^{-8A>G}$ in NCIB3610	This study
SLH38	*tyrS*$^{605G>A}$ in NCIB3610	This study
SLH57	*amyE*::P$_{tyrS}$*-lux* (from NCIB3610) in NCIB3610, Cm^{R}	This study
SLH58	*amyE*::P$_{tyrS}$*-lux* (from NCIB3610) in SLH36, Cm^{R}	This study
SLH59	*amyE*::P$_{tyrS}$*-lux* (from SLH8) in NCIB3610, Cm^{R}	This study
SLH61	*amyE*::P$_{tyrS}$*-lux* (from SLH9) in NCIB3610, Cm^{R}	This study
SLH63	*tapA-sipW-tasA*::*spec*	This study
SLH64	*tapA-sipW-tasA*::*spec*, *amyE*::*tapA-sipW-tasA*, Cm^{R}	This study
SLH65	*tapA-sipW-tasA*::*spec*, *amyE*::*tapA2-sipW-tasA*, Cm^{R}	This study
SLH66	*tapA-sipW-tasA*::*spec*, *amyE*::*tapA6-sipW-tasA*, Cm^{R}	This study
SLH69	*amyE*::P$_{hyspank}$*-tyrS*$^{-366-1269}$, Spec^{R}	This study
SLH70	*amyE*::P$_{hyspank}$*-tyrS*$^{-16-1269}$, Spec^{R}	This study
SLH75	*amyE*::P$_{tyrS}$*-lux* (no terminator) in NCIB3610, Cm^{R}	This study
RL4602	*tapA-sipW-tasA::spec, amyE::ΔtapA(13-234)-sipW-tasA,* Cm^{R}	Laboratory stock

Table S1 (Continued). Strains, plasmids, and primers used in this study.

Strain	Genotype or description	Source
Turbo *E. coli*	*recA*$^{+}$ *endA1* Δ*(hsdS-mcrB)5*	NEB
DH5α	*recA1 endA1 hsdR17* (r_K^-, m_K^+) *relA1*	Invitrogen
BL21 (De3)	F$^-$ *ompT hsdS*B (r_B^- m_B^-) *gal dcm* (DE3)	EMD Biosciences
Arctic Express (De3)	BL21 derivative for improved protein folding and solubility	Agilent
AE-pET28-BsubTyrRS-WT	Arctic Express cells harboring pSL006	This study
AE-pET28-BsubTyrRS-AV	Arctic Express cells harboring pSL007	This study
Rosetta (De3)	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3) pRARE2 (Cam^R)	EMD Biosciences

Plasmid	Genotype or description	Source
pDG364	*amyE*-integration vector, Amp^R, Cm^R	(6)
pDG1662	*amyE*-integration vector, Amp^R, Cm^R	(3)
pAH328	*sacA*-integration vector with *luxABCDE*, Amp^R, Cm^R	(2)
pAE164	*amyE*-integration vector with *tapA-sipW-tasA*, Amp^R, Cm^R	This study
pAE165	*amyE*-integration vector with *tapA*$^{558\Delta}$*-sipW-tasA*, Amp^R, Cm^R	This study
pAE166	*amyE*-integration vector with *tapA*$^{724\Omega G}$*-sipW-tasA*, Amp^R, Cm^R	This study
pLF007	*amyE*-integration vector with *luxABCDE*, Amp^R, Cm^R	This study
pSL001	*amyE*-integration vector with P_{tyrS} from NCIB3610 fused to *luxABCDE*, Amp^R, Cm^R	This study
pSL002	*amyE*-integration vector with P_{tyrS} from SLH8 fused to *luxABCDE*, Amp^R, Cm^R	This study
pSL003	*amyE*-integration vector with P_{tyrS} from SLH9 fused to *luxABCDE*, Amp^R, Cm^R	This study
pDR111	*amyE* integration vector with $P_{hyspank}$, Spec^R	(8)
pSL004	Derived from pDR111, $P_{hyspank}$-*tyrS*$^{-366-1269}$, Spec^R	This study
pSL005	Derived from pDR111, $P_{hyspank}$-*tyrS*$^{-16-1269}$, Spec^R	This study
pET-28a	T7 promoter, *lac* operator, His_6 tag (N or C)	Novagen
pSL006	Derived from pET-28a to express IPTG-inducible TyrRS-His_6 (*tyrS* from NCIB3610)	This study
pSL007	Derived from pET-28a to express IPTG-inducible TyrRS-His_6 (*tyrS* from SLH38)	This study
pSL008	*amyE*-integration vector with P_{tyrS} lacking the terminator hairpin sequence fused to *luxABCDE*, Amp^R, Cm^R	This study

Table S1 (Continued). Strains, plasmids, and primers used in this study.

Primer	Sequence	Primary Use
39	CGTTGTAAAACGACGGCCAGTGAATTCGTAGCTATGTGCGGACGGGA	amplify *trnD-Phe*
40	AACAGCTATGACCATGATTACGCCAAGCTTCATGGATCTCATAAAGTGTCAACGTATTTC	amplify *trnD-Phe*
41	CGTTGTAAAACGACGGCCAGTGAATTCGCTTTTGACAAGTCCGAAGTC	amplify *hrcA*
42	AACAGCTATGACCATGATTACGCCAAGCTTCTGATGGATATGATCAACCGCG	amplify *hrcA*
45	CGTTGTAAAACGACGGCCAGTGAATTCGCTTGGGAGATGTTGATGGC	amplify *tyrS* riboswitch
46	AACAGCTATGACCATGATTACGCCAAGCTTCGGAAGTCATCAGATGGGGAC	amplify *tyrS* riboswitch
47	CGTTGTAAAACGACGGCCAGTGAATTCCCGTCTTACAACCTTTCTGAACTGTT	amplify *ppaC*
48	AACAGCTATGACCATGATTACGCCAAGCTTCGATGAGGGGGCTGAAGATGAA	amplify *ppaC*
49	CGTTGTAAAACGACGGCCAGTGAATTCGTACTTTCACTCGTCTTCCTGAGCG	amplify *tyrS*
50	AACAGCTATGACCATGATTACGCCAAGCTTCCAGAAGCGGAAAGATCATGAGGC	amplify *tyrS*
55	GTATACCCAAACACTGCCGGCTCTC	sequence *tapA*
56	GAGCAATACTGAGCAAGACTTTGTA	sequence *tapA*
57	CCCAAGCGTGTTAAAAGACC	sequence *hrcA*
58	GCAGCAAGACGTGTTAAACT	sequence *ppaC*
59	CATCTTCAATGTCCACTGTGT	sequence *ppaC*
60	GCTCTCCGCCACTGATACTTATAC	sequence *trnD-Phe*
70	CTATTGCCGATGATAAGCTGTCAAACATGAGAATTCAGATCTGTCAACAATGGAGGAT	construct pSL001, pSL002, pSL003
71	GAGAGTCCTCCTGTCGACCTTACTAGTTATGCGGCCGCTCTAGAAATCTGGACAGCTGG	construct pSL001, pSL002, pSL003
72	GAGAGTCCTCCTGTCGACCTTACTAGTTATGCGGCCGCGGTACCACCCTACTAATAAGAG	construct pSL008
73	TTGTTTAACTTTAAGAAGGAGATATACCATGACTAACTTACTTGAAGACTTA	construct pSL006, pSL007
74	GGATCTCAGTGGTGGTGGTGGTGGTGCTCGAGTTTATACGTCACAAGGAAGT	construct pSL006, pSL007
76	TGATGACCTCGTTTCCACCGAATTAGCTTGCATGC TTATTTATACGTCACAAGGAAGT	construct pSL004, pSL005
77	TGTGTAATTGTGAGCGGATAACAATTAAGCTTAGATCTGTCAACAATGGAGGAT	construct pSL004
86	TGTGTAATTGTGAGCGGATAACAATTAAGCTTAAAGGAGCTTTATCTTATGACTAACTTAC	construct pSL005
pDR111F	TGTGTAATTGTGAGCGGATAACAATTAAGCTT	sequence pSL004, SL005
pDR111R	TGATGACCTCGTTTCCACCGAATTAGCTTGCATGC	sequence pSL004, SL005
LF002F	TGAGCGTAGCGAAAAATCCT	sequence *amyE*
LF002R	TGAGAAAATTGGGGAGGTTG	sequence *amyE*

Table S1 (Continued). Strains, plasmids, and primers used in this study.

Primer	Sequence	Primary Use
AE1	GGTAGCGACCGGCGCTCAGGATCCGATCATCATGCTGTCACCCTTTCTTTG	construct pAE164, pAE165, pAE166
AE2	GCCGATGATAAGCTGTCAAACATGAGAATTCTTAATTTTTATCCTCGCTATGCGCTTTT	construct pAE164, pAE165, pAE166
AE3	GAGGCTTGCAAATGCGATGAAAAAC	construct pAE165
AE4	GTTTTTCATCGCATTTGCAAGCCTC	construct pAE165
AE5	GAAGGAAAGCGGGGGAAGAGGATG	construct pAE166
AE6	CATCCTCTTCCCCCGCTTTCCTTC	construct pAE166

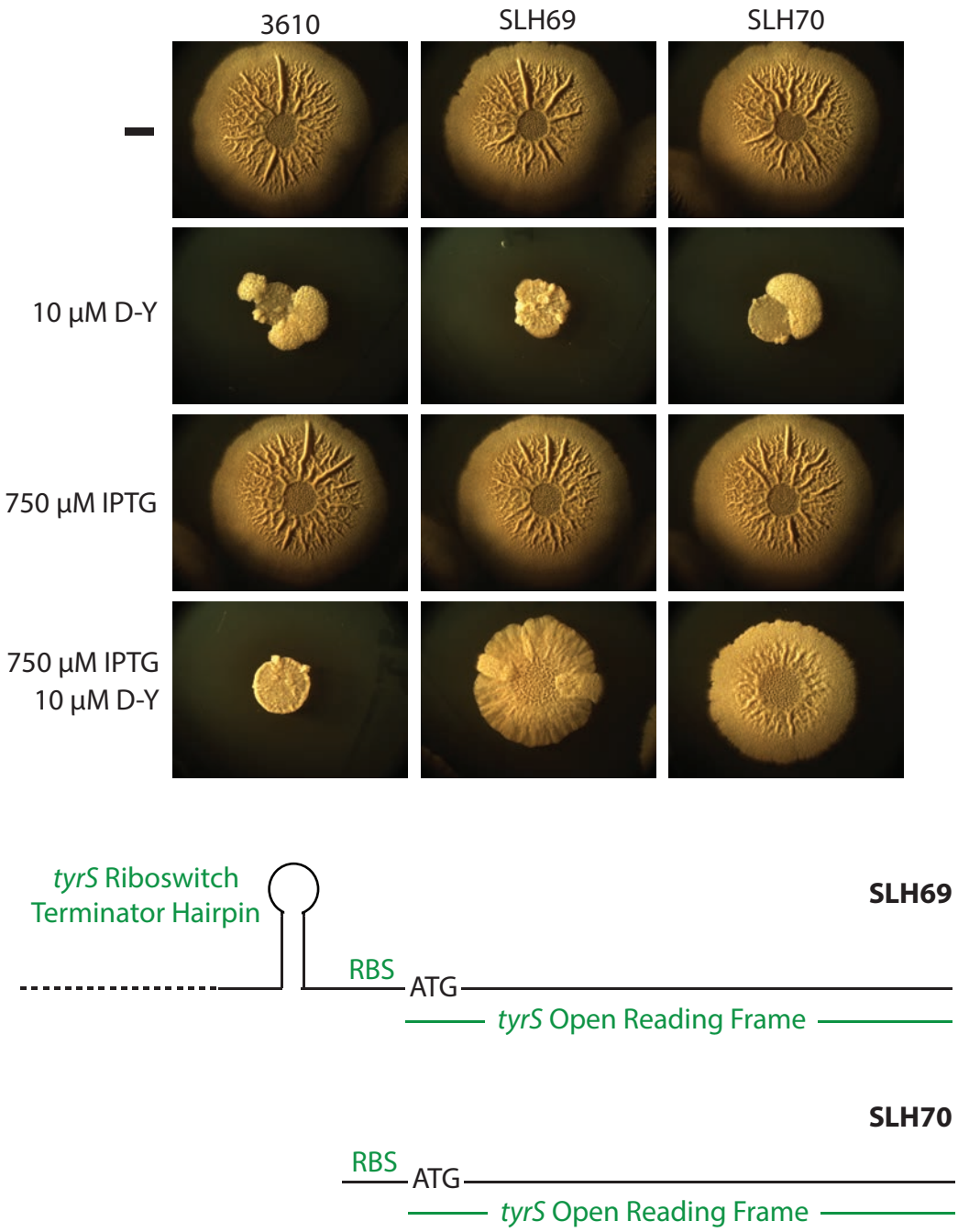

Figure S1. Overexpression of *tyrS* is sufficient to induce D-Tyr resistance. *B. subtilis* 3610, SLH69, and SLH70 were spotted on solid MSgg containing supplements as indicated. Plates were incubated at 30˚C and imaged after three days. Schematics illustrate the constructs used to overexpress the *tyrS* riboswitch, RBS, and open reading frame (in SLH69) or to overexpress the *tyrS* RBS and open reading frame (in SLH70). Both overexpression constructs were introduced at the *amyE* locus and include an IPTG-inducible promoter. The dotted line represents the *tyrS* riboswitch upstream of the terminator hairpin.

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