Supporting Information

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SI Text

SI Materials and Methods. ***Strains, media, and genetic manipulation.*** *Escherichia coli* strains DH5a and BL21 (DE3) (Novagen) were used as hosts for the construction of the mutant fragment library and for the expression of the HrdB protein, respectively. Intergeneric conjugation from *E. coli* ET12567 (pUZ8002) to *Streptomyces avermitilis* was performed as described previously (1). Manipulations of *Streptomyces* and *E. coli* were performed following standard procedures (1, 2).

Microarray construction, RNA isolation, labeling, hybridization, and scanning. A mini-DNA chip was constructed with spots, in triplicate, of 500 gene-specific oligonucleotides based on the publicly available complete genome information of *S. avermitilis*. Five milliliters of culture broths from a 96-h fermentation culture (3) were used for RNA isolation. RNA was isolated using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. The RNA was subsequently purified by QIAGEN RNeasy Mini Kit, and the quality and quantity of the purified RNA was determined by nanodrop UV spectroscopy (Ocean Optics). Then, 6 μg of RNA from 3-115 and wild-type strains were used to synthesize cDNA labeled with Cy5 and Cy3, respectively. The labeled cDNA samples were concentrated and purified by QIAquick Nucleotide Removal Kit. Hybridization was performed as described previously (4) in an Agilent Microarray Hybridization Chamber (G2534A) for 16 h at 65 °C. After hybridization, the slides were washed in Gene Expression Wash Buffer (Agilent). Microarrays were scanned on a GenePix 4000B (Axon Instruments). Microarray data extraction and analysis was performed as described previously (5).

Real-time RT-PCR analysis. Real-time RT-PCR was performed to confirm the result of transcriptional profiling. First-strand cDNA was synthesized from l μg total RNA in a 20-μL reaction volume using a cDNA synthesis kit (Takara) according to the manufacturer's instructions. Real-time PCR reactions were performed using SYBR Green I (Takara) on the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems Inc.). Gene-specific primers were designed using Primer 3.0 (Table S4). The change in fluorescence of SYBR Green I in every cycle was monitored by the system software and the threshold cycle (C_T) was measured. We used 16S rRNA as an internal control and the relative gene expression level was calculated using the formula $2^{-\Delta\Delta CT}$, where ΔC_T was the C_T value of interesting genes minus that of internal control and $\Delta\Delta C_T$ was the mean ΔC_T value of the interest strain minus that of the reference strain.

Protein expression, purification, and in vitro transcription assays. DNA encoding full-length HrdB protein (amino acids 1–512) was cloned into pET28b (Novagen) and confirmed by sequencing. *E. coli* BL21 (DE3) cells transformed with the expression plasmid were grown to log phase, induced with 0.5 mM isopropyl-1-thio-B-D-galactopyranoside, and harvested by centrifugation 5 h after induction. The cell pellets were rinsed with phosphate-buffered saline (pH 8.0, PBS) and resuspended in PBS buffer at 5 mL per gram wet weight. The cells were sonicated at 15 × 3 s with 7-s pauses at 300 W and centrifuged at 10,000 × *g* for 30 min at 4 °C. The supernatant was adjusted to the same pH and composition as the binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 8.0) and loaded onto a Ni-NTA Agarose column (GE Healthcare, HisTrap HP, No. 17-5247-01). The column was washed with five column volumes of binding buffer, followed by elution with a gradient of imidazole (50–500 mM). The eluted fractions were analyzed by gel filtration chromatography on a column (10 × 300 mm) of Superdex 75 eluted with TM buffer (20 mM Tris-HCl, pH 7.8 at 4 °C, 10 mM $MgCl_2$, 10 mM KC1, 0.1 mM EDTA) at a flow rate of 0.4 mL/ min in a FPLC system (GE Healthcare). SDS-PAGE was used to monitor the flow-through. Protein was quantified by Bradford Assay Kit (Beyotime Ltd. Co.).

DNA fragments of the promoter region were amplified from strain 3-115 genomic DNA using primers described in Table S4. DNA fragments were kept at 1.5 pmol final concentration, and σ^{hrdB} protein was added at different concentrations. Briefly, 0.25 unit of *E. coli* RNA polymerase core enzyme (EPICENTRE) mixed with 1.5 pmol σ^{hrdB} was incubated with 1.5-pmol template DNA in 16-uL transcription buffer (80 mM Hepes-KOH (pH 7.5), 32 mM $MgCl_2$, 2 mM spermidine, 40 mM DTT) at 37 °C for 20 min to form open complexes. RNA synthesis was initiated by adding 4 uL of NTP-heparin mixture (0.32 mM ATP, CTP, GTP; 0.1 mM UTP; 3 mg heparin; and 2 μCi [α-^{32}P]UTP). After 20-min incubation at 37 °C, transcripts were separated in 7.5 M urea polyacrylamide gel electrophoresis and detected by autoradiography.

Library construction, high-throughput screening (HTS) screening, and titer measurements. The *hrdB* gene with its native promoter region was amplified from *S. avermitilis* ATCC31267 genomic DNA using primers XAPl and EAP2. The amplified fragment, confirmed by sequencing, was digested by *Xba*I and *EcoR*I and inserted into pSET152 to generate pZY126. Fragment mutagenesis was performed using the GeneMorph® II EZClone Domain Mutagenesis Kit (Stratagene) as described in the manufacturer's instructions with the following modifications: 100–500 ng of pZY126 DNA was used as the initial template, and the DNA coding the full-length HrdB protein was amplified using primers AP3 and AP4. Plasmids containing mutant *hrdB* genes were directly transformed into competent *E. coli* ET12567 (pUZ8002) cells. Cells were spread on LB-agar plates containing 50 μg/mL apramycin and 25 μg/mL chloramphenicol. Clones that survived antibiotic selection were scraped off to create a liquid library. The plasmid library was conjugation-transferred into strain 3-115 following standard procedures.

The HTS screening, fermentation (Erlenmeyer flasks and fermentor), and avermectin analysis of mutants were performed as described previously (6, 7). The parental strain 3-115 was simultaneously cultured, and its production was used as a control. The production of avermectins in strain Cl containing only a blank pSET152 and in strain C2 with an additional copy of the *hrdB* gene was also investigated. The stability of those yield-improved strains were tested through three iterations of shake flask fermentation.

Fragment rescue and sequence analysis. Genomic DNA of the yield-improved strains was isolated and completely digested by *Pst*I; the digested fragments containing mutant *hrdB* gene were purified and self-ligated. The self-ligation products were introduced into *E. coli*, and colonies that survived apramycin selection were selected. Plasmids from those colonies were digested by *Xba*I and *EcoR*I and inserted into pSET152 to generate pSET152::*hrdBm*, which was introduced into *E. coli* ET12567 (pUZ8002) and then conjugationally transferred into 3-115. The fermentation and avermectin production analysis of these transformants were performed and analyzed. At the same time, the *Xba*I- *EcoR*I

fragments were inserted into pUC18 for sequencing. The predicted amino acid sequences were aligned with the wild-type HrdB^{ATCC31267}, and mutant sites were shown in the corresponding location. The conserved functional domains proposed by Marcos (8) were also presented. Sequences were aligned and compared using Clustal W version 1.82.

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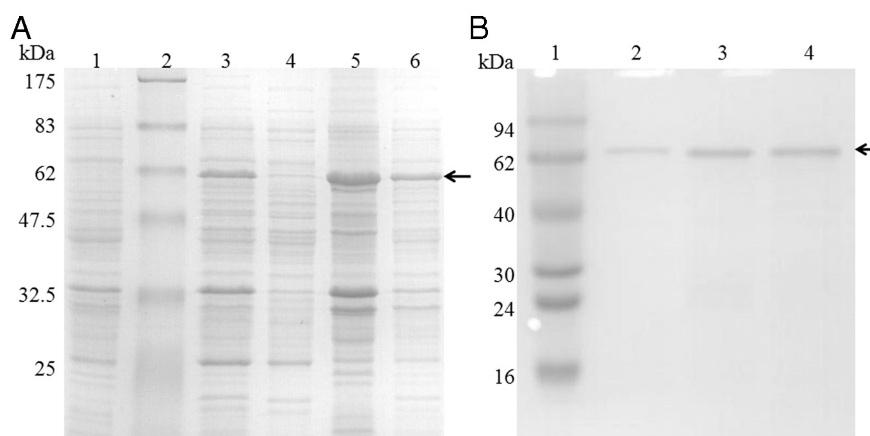

Fig. S1. Expression and purification of recombinant His6-HrdB protein. (*A*) SDS-PAGE. Lane 1, lysate from uninduced cells; lane 2, protein marker (P7708G, NEB); lane 3, lysate from cells induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside at 25 °C for 4 h; lane 4, supernatant of the uninduced cells sonicated on ice and centrifuged at 10,000 × *g* for 30 min; lanes 5 and 6, pellet and supernatant, respectively, of the induced cell in the same treatment with lane 3. (*B*) Purification under native conditions purified using Ni-NTA Agarose with the indicated imidazole concentrations in the elution steps. Lane 1, protein marker (DM101, Transgen); lanes 2, 3, and 4, 50-mM, 100-mM, and 150-mM elution, respectively. The arrow points to the band corresponding to the HrdB protein.

Table S1. Genes with at least a twofold higher expression level in the overproducer *S. avermitilis* 3-115

Accession number	Gene	Function	Ratio(3-115 / 31267)	Candidate for HrdB
Sig-regulons				
SAV4303	*sig37*	RNA polymerase ECF-subfamily sigma factor	2.02	+
SAV3843		Anti-sigma factor antagonist	2.29	–
SAV424	*sig3*	RNA polymerase ECF-subfamily sigma factor	2.36	+
SAV3012	*rsbW*	Anti-sigma factor	2.74	–
Gene transcription and regulation				
SAV736		Putative transcriptional regulator	2.22	–
SAV520		Putative transcriptional regulator	2.67	–
SAV1728		LacI-family transcriptional regulator	2.76	–
SAV143		Putative TetR-family transcriptional regulator	2.86	–
SAV551		Putative TetR-family transcriptional regulator	2.97	+
SAV2785		Putative regulatory protein	2.61	–
SAV537		Putative Sir2-family regulator protein	2.17	–
SAV4703		Two-component system sensor kinase	2.22	–
SAV4704		Two-component system response regulator	2.12	–
SAV6039		Two-component system sensor kinase	2.14	–
SAV129		Two-component system response regulator	3.06	–
Avermectin biosynthesis				
SAV953	*aveG*	Thioesterase	2.92	–
SAV935	*aveR*	LuxR-family transcriptional regulator	2.74	+
SAV940	*aveC*	CHC-B2:CHC-B1 ratio	2.12	–
SAV937	*aveD*	C5-O-methyltransferase	2.01	–
SAV938	*aveAI*	Type I polyketide synthase AVES 1	2.87	–
SAV942	*aveAIII*	Type I polyketide synthase AVES 3	2.03	–
SAV947	*aveBIII*	Glucose-1-phosphate thymidyltransferase	2.48	–
SAV948	*aveBIV*	dTDP-4-keto-6-deoxy-L-hexose 4-reductase	2.02	–
SAV949	*aveBV*	dTDP-4-keto-6-deoxyhexose 3,5-epimerase	2.20	–
Energy metabolism				
SAV1818	*sucC1*	Putative succinyl-CoA synthetase beta subunit	4.18	–
SAV2824	*ackA*	Putative acetate kinase	4.72	–
SAV3566	*ppc*	Putative phosphoenolpyruvate carboxylase	2.86	–
Transport				
SAV4076	*pitH1*	Low-affinity inorganic phosphate transporter	2.16	–
SAV2185		ABC transporter ATP-binding protein	2.76	–
Amino acid metabolism				
SAV2918	*thrA*	Putative homoserine dehydrogenase	2.09	–
Morphological differentiation				
SAV4604	*ssgB*	Morphological differentiation-associated protein	2.04	+

Table S2. Avermectin biosynthetic genes and their comparative expression levels

ORF	Gene / Function	Ratio (3-115 / ATCC 31267)
SAV935	*aveR* (LuxR-family transcriptional regulator)	2.74
SAV936	*aveF* (C-5 ketoreductase)	1.02
SAV937	*aveD* (C5-O-methyltransferase)	2.01
SAV938	*aveAI* (type I polyketide synthase AVES 1)	2.87
SAV939	*aveAII* (type I polyketide synthase AVES 2)	0.94
SAV940	*aveC* (CHC-B2:CHC-B1 ratio)	2.12
SAV941	*aveE* (cytochrome P450 hydroxylase)	0.60
SAV942	*aveAIII* (type I polyketide synthase AVES 3)	2.03
SAV943	*aveAIV* (type I polyketide synthase AVES 4)	0.97
SAV944	orf-1 (reductase)	1.62
SAV945	*aveBI* (glycosyl transferase)	0.36
SAV946	*aveBII* (dTDP-glucose 4,6-dehydratase)	0.30
SAV947	*aveBIII* (glucose-1-phosphate thymidyltransferase)	2.48
SAV948	*aveBIV* (dTDP-4-keto-6-deoxy-L-hexose 4-reductase)	2.02
SAV949	*aveBV* (dTDP-4-keto-6-deoxyhexose 3,5-epimerase)	2.20
SAV950	*aveBVI* (dTDP-4-keto-6-deoxy-L-hexose2,3-dehydratase)	0.90
SAV951	*aveBVII* (dTDP-6-deoxy-L-hexose 3-O-methyltransferase)	0.72
SAV952	*aveBVIII* (dTDP-4-keto-6-deoxy-L-hexose 2,3-reductase)	0.69
SAV953	*aveG* (thioesterase)	2.92

Table S3. Comparison of the production of a strain containing mutant *hrdB* rescued from the yield-improved strains against 3-115

	Relative titer of avermectin B1a (%)
3-115	100
A56	152.20 ± 3.87
A393	146.11 ± 3.42
pZY148-*hrdB*^{A56}	148.23 ± 2.36
pZY150-*hrdB*^{A393}	144.65 ± 3.56

Five transformants of the pZY148-*hrdB*^{A56} and pZY150-*hrdB*^{A393} were randomly picked for analysis.

Table S4. Primers used in this study

Gene	Length (bp)	Primer	Sequence (5′-3′)
RT-PCR analysis			
aveR	158	aveR-F	AGGGAGTTTCCTGTCTGCAC
		aveR-R	ACTCTTTCCACAGCCCATTC
aveAI	223	aveAI-F	ACCAGCAACCCAGACCAAC
		aveAI-R	CGGAGGAGTAGAGGACGAAG
aveAIII	243	aveAIII-F	AGACGTGACCCTTCAGGTTG
		aveAIII-R	TGATCGTAAGCCGTGTTCAG
16S rRNA	225	rRNA-F	CTCGTGTCGTGAGATGTTGG
		rRNA-R	TGAGATTCGCTCCACCTTG
In vitro transcription assay			
*rrnD*p	629	AvrrnDR	GATGTGCGTTGCCCTCCG
		AvrrnDF	CCAGCGTTCGTCCTGAGCCA
*aveR*p	452	EM06F	CCCTCTGGACCCTTGCTCG
		EM06R	GGTGCAGACAGGAAACTCCCT
*aveAI*p	354	EM07F	GGTCGGGAACCTCCGCAATC
		EM07R	GCCGTCCATCCTCTGCACCT
Library construction			
*hrdB*1	2352	XAP1	CACTCTAGACCCTGAGGTGGAGCGTGTG
		EAP2	CTCGAATTCGGTCATGGAATACCCAGAGTGAT
hrdB	1560	AP3	TGTTCGTGTCGGCCAGCAC
		AP4	TGCGTACAGCCGAGACCTAGTC