Supplementary information for

Regulation of the macrolide resistance ABC-F translation factor MsrD

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Supplementary Table 1. Minimum inhibitory concentration (MIC) and half maximal inhibitory concentration (IC_{50}) of *E. coli* DB10 expressing *msrD* variants in presence of erythromycin. See Methods for experimental details. MIC values exceeding control plasmid are shown in bold.

	Erythromycin	
***E. coli* DB10**	**MIC (μM)**	**IC_{50} (μM)**
pBAD-*Control*	2	0,179 ± 0,007
pBAD-$msrD_{WT}$	**16**	4,592 ± 0,582
pBAD-$msrD_{EQ2}$	2	0,267 ± 0,027
pBAD-$msrD_{E125Q}$	**4**	1,427 ± 0,147
pBAD-$msrD_{E434Q}$	2	0,342 ± 0,04
pBAD-$msrD_{\Delta Loop}$	2	0,209 ± 0,025
pBAD-$msrD_{\Delta PtlM}$	2	0,295 ± 0,048
pBAD-$msrD_{R241A}$	**16**	3,632 ± 0,316
pBAD-$msrD_{L242A}$	**8**	1,621 ± 0,152
pBAD-$msrD_{H244A}$	**8**	1,492 ± 0,126
pBAD-$msrD_{H244W}$	**4**	0,446 ± 0,057

Supplementary Table 2. Cryo-EM data collection and refinement statistics.

	Erythromycin-stalled *Escherichia coli* 70S ribosome with streptococcal MsrDL nascent chain (PDB 7Q4K, EMD-13805, EMD-13806, EMD-13807, EMD-13808)
Data collection and processing	
Microscope	FEI Talos Arctica (IECB, Pessac, France)
Detector	K2 Summit direct electron detector (Gatan)
Magnification (X)	120,000
Voltage (kV)	200
Electron exposure (e^{-}/Å^2)	64
Defocus range (μm)	-0.5 to -2.7
Pixel size (Å)	1.2
Symmetry imposed	C1
Initial particle images (no.)	158,200
Final particle images (no.)	62,093
Map resolution (Å)	70S ribosome (EMD-13805): 3 50S subunit (EMD-13806): 2.97 30S subunit Body (EMD-13807): 3.08 30S subunit Head (EMD-13808): 3.3
FSC threshold	0.143
Model building and refinement	
Initial model (PDB code)	6TC3
Model resolution (Å)	2.7
FSC threshold	0.143
Model resolution range (Å)	2.5 – 8.7
Model composition	-
Non-hydrogen atoms	146,618
Protein residues	5,682
RNA bases	4,710

Ligands	173
B-factor (Å^2)	-
Protein (min./max./mean)	31.70/183.09/69.05
Ligands (min./max./mean)	20.00/458.82/75.79
R.m.s. deviations	-
Bond lengths (Å)	0.015
Bond angles (°)	1.648
Validation	-
MolProbity score	1.49
Clashscore	3.83
Poor rotamers (%)	0.63
Ramachandran plot	-
Favored (%)	95.42
Allowed (%)	4.45
Disallowed (%)	0.13

Supplementary Table 3. Strains and plasmids used in this study.

Bacterial strains	**References**
E. coli DB10 (Derived from *E. coli* PR7)	[1]
E. coli K12 MG1655	N/A
Plasmids	**References**
pBAD-*Control* (pBAD33)	[2]
pBAD-$msrD_{WT}$ (pVN50)	[2]
pBAD-$msrD_{EQ2}$	This study
pBAD-$msrD_{E125Q}$	This study
pBAD-$msrD_{E434Q}$	This study
pBAD-$msrD_{\Delta Loop}$	This study
pBAD-$msrD_{\Delta PtlM}$	This study
pBAD-$msrD_{R241A}$	This study
pBAD-$msrD_{L242A}$	This study
pBAD-$msrD_{H244A}$	This study
pBAD-$msrD_{H244W}$	This study
pMMB-67EH	[3]
pMMB-67EH-*yfp*	This study
pMMBpLlacO-1-67EH-*yfp*	This study
pMMB-*msrDL*-$msrD_{(1-3)}$:*yfp*	This study
pMMB-$msrDL_{(no\_ORF)}$-$msrD_{(1-3)}$:*yfp*	This study
pMMB-$msrDL_{(no\_term)}$-$msrD_{(1-3)}$:*yfp*	This study
pMMB-$msrDL_{(Y2A)}$-$msrD_{(1-3)}$:*yfp*	This study
pMMB-$msrDL_{(L3A)}$-$msrD_{(1-3)}$:*yfp*	This study
pMMB-$msrDL_{(I4A)}$-$msrD_{(1-3)}$:*yfp*	This study
pMMB-$msrDL_{(F5A)}$-$msrD_{(1-3)}$:*yfp*	This study
pMMB-$msrDL_{(M6A)}$-$msrD_{(1-3)}$:*yfp*	This study
pMMB-$msrDL_{(UAA>UGA)}$-$msrD_{(1-3)}$:*yfp*	This study
pMMB-$msrDL_{(UAA>UAG)}$-$msrD_{(1-3)}$:*yfp*	This study
pMMB-$msrDL_{(WT\text{-}isocodons)}$-$msrD_{(1-3)}$:*yfp*	This study
pMMB-$msrDL_{(MYLIFMA\text{-}isocodons)}$-$msrD_{(1-3)}$:*yfp*	This study

Supplementary Table 4. Oligonucleotides used in this study.

No.	Name	Sequence (5' → 3')	Purpose
pBAD plasmids			
1	msrD_F	ATGGAATTAATATTAAAAGCAAAAGACATTCGTGTGG	Amplification of *msrD*$_{WT}$
2	msrD_R	TTAGTGATGGTGATGGTGATGTTTCAGATTTATTTTCTTATC	
3	pBAD_F	CATCACCATCACCATCACTAATCTAGAGTCGACCTGCAGGC	Amplification of pBAD backbone
4	pBAD_R	GCTTTTAATATTAATTCCATGGTGAATTCCTCCTGCTAGCC	
5	msrD$_{EQ2}$_F1	GGTATTTTAGCGGATCAACCTACGAG	*msrD* mutagenesis
6	msrD$_{EQ2}$_R1	GGAAGTTACTGGGTTGATCCATTATTAG	
7	msrD$_{EQ2}$_F2	AACCCAGTAACTTCCTTGACATACC	
8	msrD$_{EQ2}$_R2	GATCCGCTAAAATACCATGAACC	
9	msrD$_{E125Q}$_F	GGTATTTTAGCGGATCAACCTACGAGCCATTTAG	
10	msrD$_{E125Q}$_R	GGCTCGTAGGTTGATCCGCTAAAATACCATG	
11	msrD$_{E434Q}$_F	CTAATAATGGATCAACCCAGTAACTTCCTTGAC	
12	msrD$_{E434Q}$_R	GGAAGTTACTGGGTTGATCCATTATTAGGATG	
13	msrD$_{\Delta Loop}$_F	GGAAAGGGCTGCGGAGGAAAAGGGAGGAGGAAAGATGTATAATGCTGCTAAAAC	
14	msrD$_{\Delta Loop}$_R	GCAGCATTATACATCTTTCCTCCTCCCTTTTCCTCCGCAGCCCTTTCCAATCGGG	
15	msrD$_{\Delta PtlM}$_F	CTGATTATCTTCGTCAGAAAGGAGGAGGACCGGAAGGCATTCGCAGAATTCG	
16	msrD$_{\Delta PtlM}$_R	CGAATTCTGCGAATGCCTTCCGGTCCTCCTCCTTTCTGACGAAGATAATCAGAATAG	
17	msrD$_{R241A}$_F	GAAGACGGAGGGGCTTTAGCTCATCAAAAATC	
18	msrD$_{R241A}$_R	GATGAGCTAAAGCCCCTCCGTCTTCAGTAC	
19	msrD$_{L242A}$_F	GACGGAGGGCGTGCAGCTCATCAAAAATCAATAG	
20	msrD$_{L242A}$_R	GATTTTTGATGAGCTGCACGCCCTCCGTCTTCAG	
21	msrD$_{H244A}$_F	GCGTTTAGCTGCTCAAAAATCAATAGGAAGTAAGG	

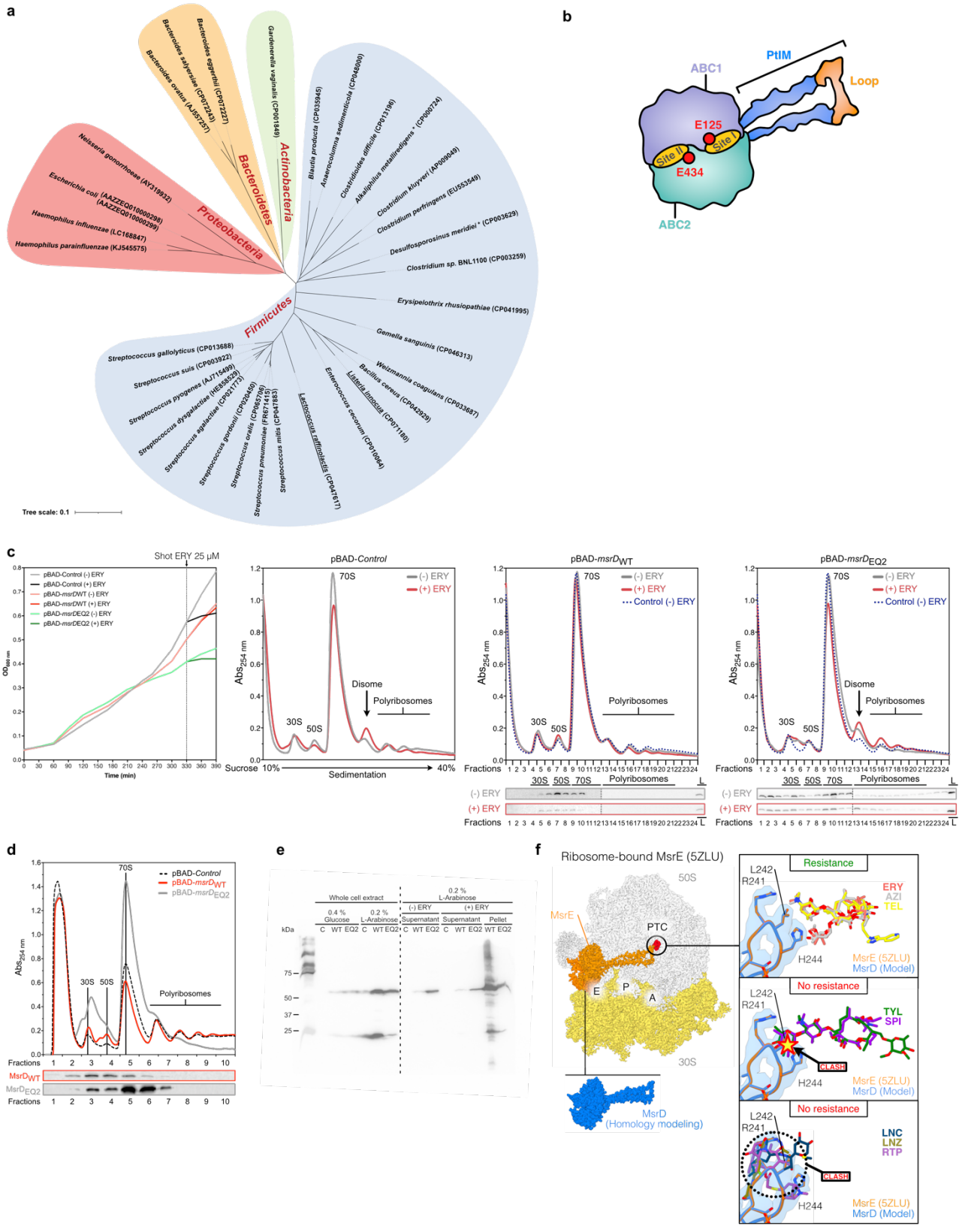
22	$msrD_{H244A}$_R	CTATTGATTTTTGAGCAGCTAA ACGCCCTCCGTCTTC	
23	$msrD_{H244W}$_F	GCGTTTAGCTTGGCAAAAATCA ATAGGAAGTAAGG	
24	$msrD_{H244W}$_R	CTATTGATTTTTGCCAAGCTAA ACGCCCTCCGTCTTC	
pMMB plasmids			
25	pMMB-PlacO_F	GTATAAATGTGAGCGGATAAC ATTGACATTGTGAGCGGATAA CAAGATACTGAGCACATCACA CAGGAAACAGAATATGTCC	Replacement of P_{tac} promoter by P_{lacO} [4]
26	pMMB-PlacO_R	GTTATCCGCTCACATTTATACA GCTCATTTCAGAATATTTGCC	
27	msrDL-$msrD_{(1-3)}$:yfp_F1	CGCAGGGTTTTCCCTGCATAC AAGCAAATGAAAGCATGCGAT TATAGACAGGAGGAAATGTTAT GGAATTAATCGTAAAAATCGTG AGCAAGG	Fusion of *msrDL*-$msrD_{(1-3)}$ cistron to *yfp*
28	msrDL-$msrD_{(1-3)}$:yfp_F2	CTGAGCACAACAATATTGGAG GAATATTTATGTATCTTATTTTC ATGTAACTCTTCCTGCTAAAAT CGCAGGGTTTTCCCTGCATAC AAGC	
29	yfp_R	TTACTTGTACAGCTCGTCCATG CCGAGAGTGATCCCGGCGGC GG	
30	pMMB-backbone_F	CATGGACGAGCTGTACAAGTA ATAATTCGAGCTCGGTACCCG GG	-
31	pMMB-backbone_R	CCTCCAATATTGTTGTGCTCAG TATCTTGTTATCCGCTCACAAT GTC	-
32	pMMB-control_F	CAACAATATTGGAGGAATATTT TAATTCGAGCTCGGTACCC	-
33	pMMB-control_R	GGGTACCGAGCTCGAATTAAA ATATTCCTCCAATATTGTTG	-
34	$msrDL_{(no_term)}$_F	ATGTATCTTATTTTCATGTAACT CTCCCTGCATACAAGCAAATG	Deletion of RIT
35	$msrDL_{(no_term)}$_R	AGAGTTACATGAAAATAAGATA CATAAATATTCCTCC	
36	$msrDL_{(no_ORF)}$_F	CAACAATATTGGAGGAATATTT TAGTATCTTATTTTCATGTAACT CTTCC	Suppression of ORF *msrDL*
37	$msrDL_{(no_ORF)}$_R	GGAAGAGTTACATGAAAATAA GATACTAAAATATTCCTCCAAT ATTGTTG	
38	$msrDL_{Y2A}$_F	CAATATTGGAGGAATATTTATG GCACTTATTTTCATGTAACTCT TCC	*msrDL* mutagenesis
39	$msrDL_{Y2A}$_R	GGAAGAGTTACATGAAAATAA GTGCCATAAATATTCCTCCAAT ATTG	
40	$msrDL_{L3A}$_F	TTGGAGGAATATTTATGTATGC AATTTTCATGTAACTCTTCC	

41	msrDL$_{L3A}$_R	GGAAGAGTTACATGAAAATTG CATACATAAATATTCCTCCAA	
42	msrDL$_{I4A}$_F	GGAGGAATATTTATGTATCTTG CATTCATGTAACTCTTCCTG	
43	msrDL$_{I4A}$_R	CAGGAAGAGTTACATGAATGC AAGATACATAAATATTCCTCC	
44	msrDL$_{F5A}$_F	GGAATATTTATGTATCTTATTG CAATGTAACTCTTCCTG	
45	msrDL$_{F5A}$_R	CAGGAAGAGTTACATTGCAATA AGATACATAAATATTCC	
46	msrDL$_{M6A}$_F	ATTTATGTATCTTATTTTCGCAT AACTCTTCCTGCTAAAATCGCA GG	
47	msrDL$_{M6A}$_R	CCTGCGATTTTAGCAGGAAGA GTTATGCGAAAATAAGATACAT AAAT	
48	msrDL$_{TAG}$_F	GTATCTTATTTTCATGTAGCTC TTCCTGCTAAAATCG	
49	msrDL$_{TAG}$_R	CGATTTTAGCAGGAAGAGCTA CATGAAAATAAGATAC	
50	msrDL$_{TGA}$_F	GTATCTTATTTTCATGTGACTC TTCCTGCTAAAATCG	
51	msrDL$_{TGA}$_R	CGATTTTAGCAGGAAGAGTCA CATGAAAATAAGATAC	
52	msrDL$_{MYLIFMA}$-isocodons_F	ATGTACCTGATCTTCATGGCCT AACTCTTCCTGCTAAAATCGCA GGG	
53	msrDL$_{MYLIFMA}$-isocodons_R	TTAGGCCATGAAGATCAGGTA CATAAATATTCCTCCAATATTG TTGTGCTCAGTATCTTGTTATC CGC	
54	msrDL$_{WT}$-isocodons_F	ATTTATGTACCTGATCTTCATG TAACTCTTCCTGCTAAAATCGC	
55	msrDL$_{WT}$-isocodons_R	GCGATTTTAGCAGGAAGAGTT ACATGAAGATCAGGTACATAAA T	
In vitro transcription and translation			
56	T7_pMMB_F	GCGAATTAATACGACTCACTAT AGGGAGCGGATAACAAGATAC TGAGCAC	-
57	TP_msrDL_R	GGTTATAATGAATTTTGCTTAT TTAATTCCATAACATTTCCTCC	-
58	TP_NV1_R	GGTTATAATGAATTTTGCTTAT T	CY5 chromophore modification in 5’
Northern blot probe			
59	pMMB_3UTR	CAGCCAAGCTTGCATGCCTGC AGGTCGACTCTAGAGGATCCC CGGG	-
Cryo-EM			
60	cryoEM-msrDL_R	GCAGGGAAAACCCTGCG	-

Supplementary Table 5. DNA templates used in this study.

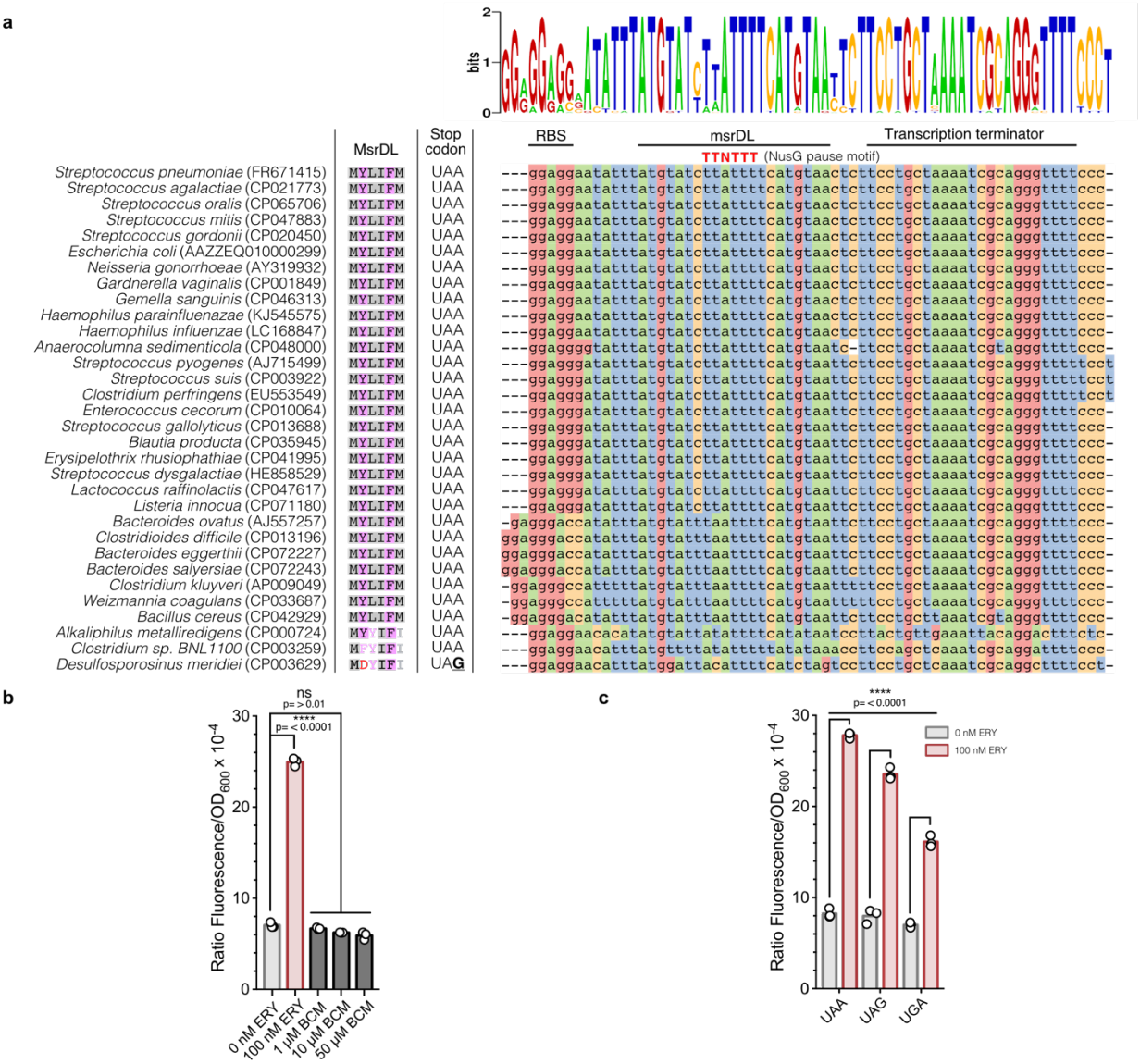
No.	Name	Sequence (5' → 3')
1	TP_msrDL$_{WT}$	GCGAATTAATACGACTCACTATAGGGAGCGGATAACAAGA TACTGAGCACAACAATATTGGAGGAATATTTATGTATCTTA TTTTCATGTAACTCTTCCTGCTAAAATCGCAGGGTTTTCCC TGCATACAAGCAAATGAAAGCATGCGATTATAGACAGGAG GAAATGTTATGGAATTAAATAAGCAAAATTCATTATAACC
2	TP_msrDL$_{7A\text{-}iso}$	GCGAATTAATACGACTCACTATAGGGAGCGGATAACAAGA TACTGAGCACAACAATATTGGAGGAATATTTATGTACCTGA TCTTCATGGCCTAACTCTTCCTGCTAAAATCGCAGGGTTTT CCCTGCATACAAGCAAATGAAAGCATGCGATTATAGACAG GAGGAAATGTTATGGAATTAAATAAGCAAAATTCATTATAA CC
3	CryoEM_*msrDL*	GCGAATTAATACGACTCACTATAGGGAGCGGATAACAAGA TACTGAGCACAACAATATTGGAGGAATATTTATGTATCTTA TTTTCATGTAACTCTTCCTGCTAAAATCGCAGGGTTTTCCC TGC

SUPPLEMENTARY FIGURE 1



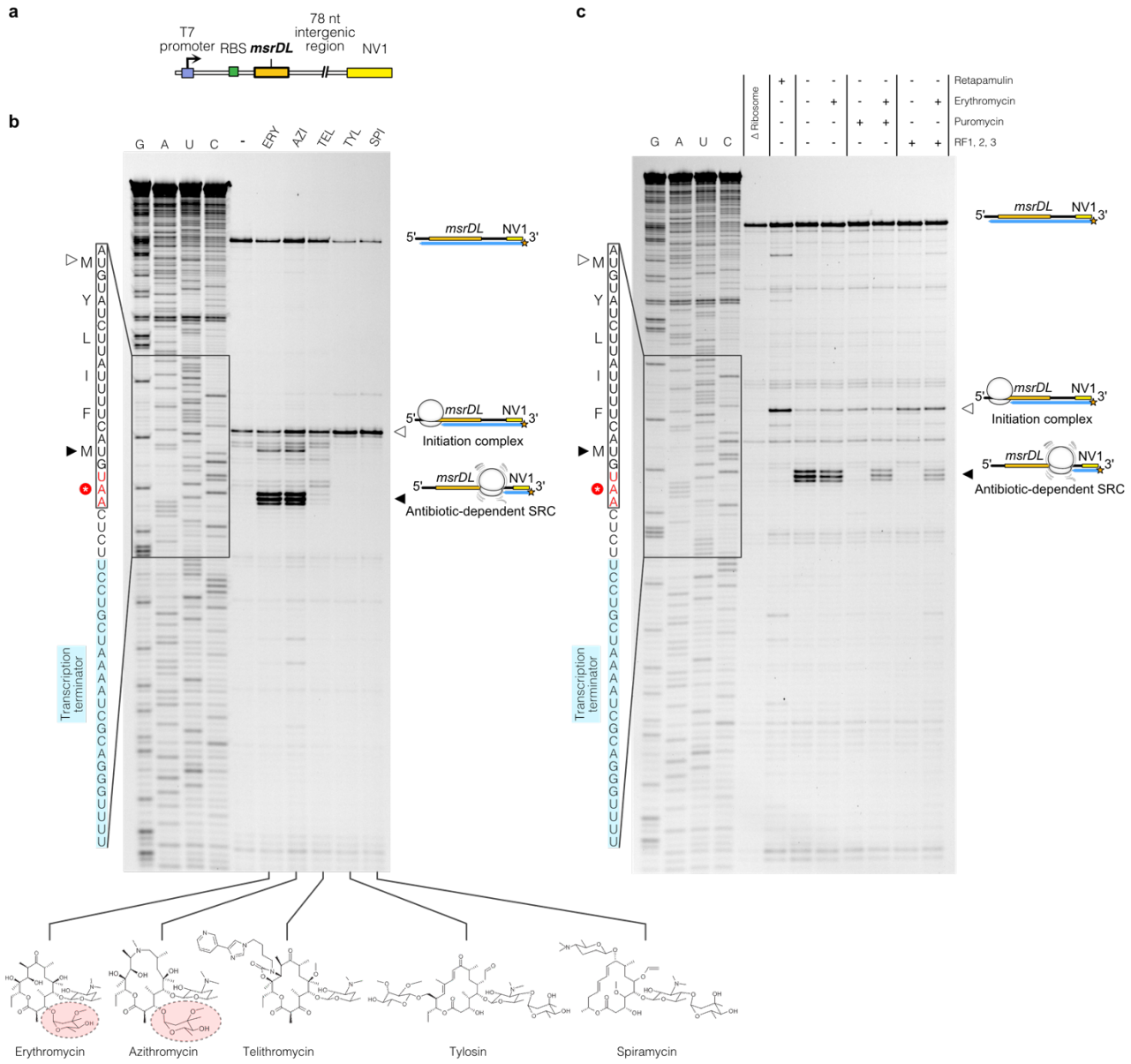
Supplementary figure 1. *In vivo* characterization of MsrD. (a) Dissemination of the *mefA/msrD* macrolide resistance operon among a wide range of non-pathogenic and pathogenic bacterial species. The *mefA/msrD* operon (Genbank accession No. FR671415) was blasted and identified in indicated species, integrated to the genome. Species where the operon was found on plasmid are underlined. Species where *msrD* was found to disseminate alone with *msrDL* are indicated by an asterisk. Corresponding Genebank accession numbers are indicated between brackets. To generate the tree, 16S rRNA sequences were retrieved, aligned using Clustal W [5], and resulting cladogram was adapted on iTOL (https://itol.embl.de/). Colored zones indicate bacterial phyla. (b) Schematic of MsrD illustrating main features of ABC-F translation factors and the position of ATP hydrolysis Site I and Site II whose catalytic residues are respectively E125 and E434. Colors are the same as Fig. 1b. (c) Growth curves, polyribosomes analysis and western blotting of *E. coli* DB10 strain expressing *msrD* (pBAD-$msrD_{WT}$), $msrD_{EQ2}$ (pBAD-$msrD_{EQ2}$) or a control (pBAD-*Control*) unexposed or exposed to 25 µM of ERY during one hour (Cells were treated after 330 min as indicated by "Shot ERY 25 µM"). "L" stands for total lysate. Note that these sample were precipitated with trichloroacetic acid (d) Polyribosomes analysis and western-blotting of *E. coli* K12 MG1655 expressing *msrD*WT (pBAD-*msrD*WT), *msrD*EQ2 (pBAD-*msrD*EQ2) or a control (pBAD-*Control*). As no erythromycin was added, 100 $\mu g.ml^{-1}$ chloramphenicol was added to stabilize translating ribosomes. (e) Solubility assay shows that $MsrD_{WT}$ tends precipitate in insoluble fraction while $MsrD_{EQ2}$ tends to stay more soluble. *E. coli* DB10 strain containing pBAD-*Control* ("C"), pBAD-$msrD_{WT}$ (WT) or pBAD-$msrD_{EQ2}$ ("EQ2") was inoculated at OD_{600}=0.1 and grew in presence of 0.4 % Glucose or 0.2 % L-Arabinose at 37 °C under vigorous shaking for 6 hours. Cultures were normalized, centrifuged and resuspended (1 ml at OD_{600}=1 was resuspended in 30 µL Laemmli 1X). In total, 5 µL of whole cell extract were loaded on SDS-PAGE gel. In parallel, cultures similar to those realized for polyribosome analysis presented in Fig. 1c and 1d were done. Briefly, cells were inoculated at OD_{600}=0.1 and grew in presence of 0.2 % L-Arabinose at 37 °C under vigorous shaking. During mid-exponential phase, 25 µM ERY was added or not. Cells were harvested and lysed after 1 h, lysate being clarified by centrifugation from insoluble fraction. After normalization of lysate, a total of 10 µg of RNAs were load on SDS-PAGE gel. Western-blotting was performed as explained in Methods. (f) Steric occlusion mechanism by MsrD is incompatible with its resistance profile. A homology model of MsrD was generated using SWISS-MODEL [6] and aligned to ribosome-bound MsrE structure (PDB 5ZLU) [7]. Antibiotics to which MsrD provides resistance (ERY PDB: 6ND6, red; AZI PDB: 4V7Y, gray; TEL PDB: 4V7Z, yellow) or not (TYL PDB: 1K9M, green; SPI PDB: 1KD1, violet; LNC PDB: 5HKV, dark teal; LNZ PDB: 3CPW, khaki; RTP PDB: 2OGO, purple) were aligned based on domain V of 23S rRNA [8–13]. Density for MsrE is shown in pale blue, and conserved residues R241A, L242A and H244A are indicated.

SUPPLEMENTARY FIGURE 2



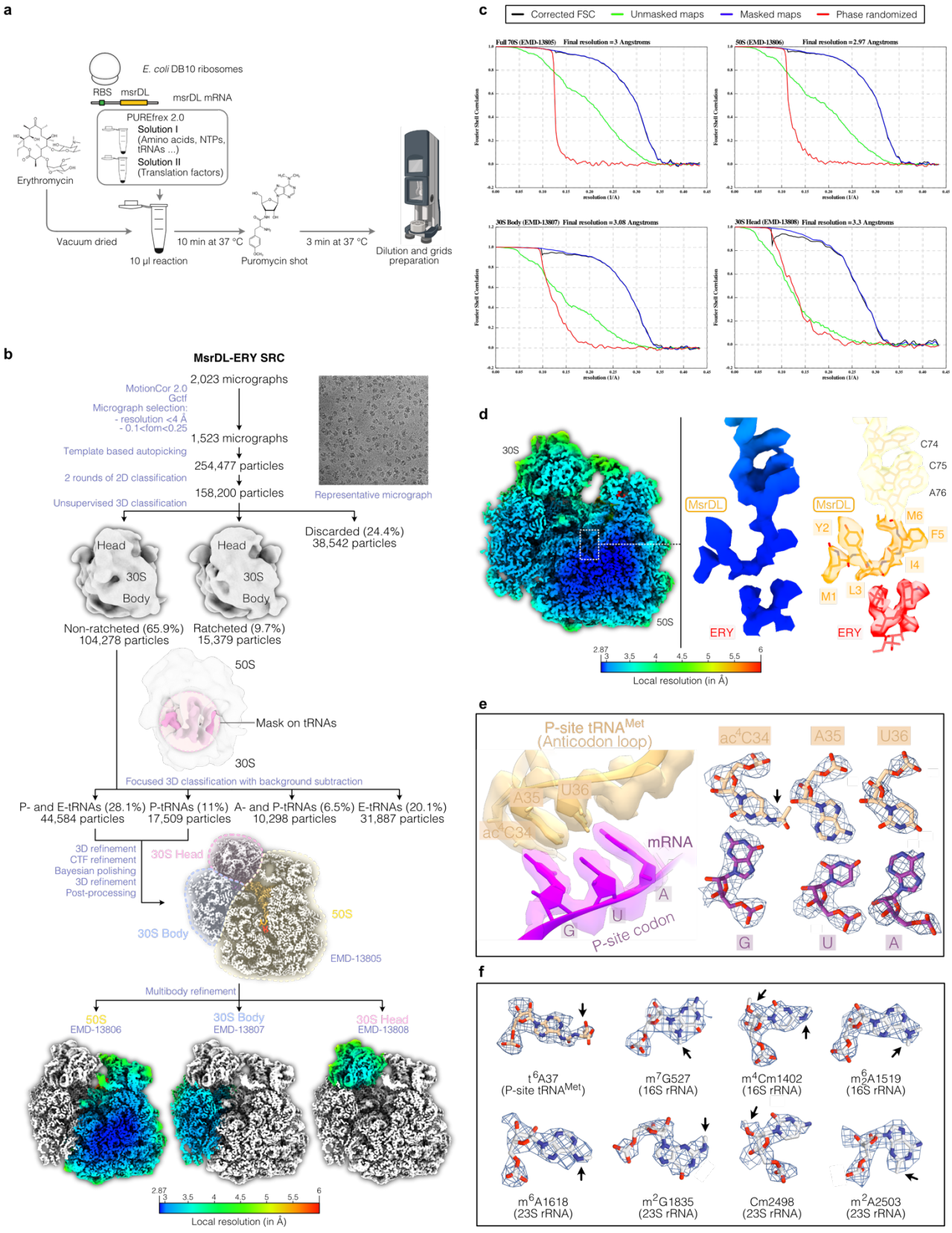
Supplementary figure 2. Rho-independent transcription termination regulates *msrD* expression. (a) Conservation of *msrDL*, NusG-dependent RNAP pausing site and its rho-independent transcription terminator. Sequences corresponding to Fig. 1a were aligned with Clustal W [5], and visualized with JalView according to nucleotide [14]. Genbank accession numbers are indicated between brackets. Logo was generated using WebLogo (https://weblogo.berkeley.edu/logo.cgi). (b) Bicyclomycin (BCM) failed to constitutively induce *msrD*$_{(1\text{-}3)}$:*yfp* expression in absence of ERY after 17 h, demonstrating that *msrD* is not regulated by a Rho-dependent terminator. Error bars represent mean ± s.d. for triplicate experiments. (c) Effects of *msrDL* stop codon mutation on the expression of *msrD*$_{(1:3)}$:*yfp*. Bacteria were grown during 17 h in presence of 1 mM IPTG, in the absence (grey histograms) or in the presence of 100 nM ERY (red histograms). Error bars represent mean ± s.d. for triplicate experiments.

SUPPLEMENTARY FIGURE 3



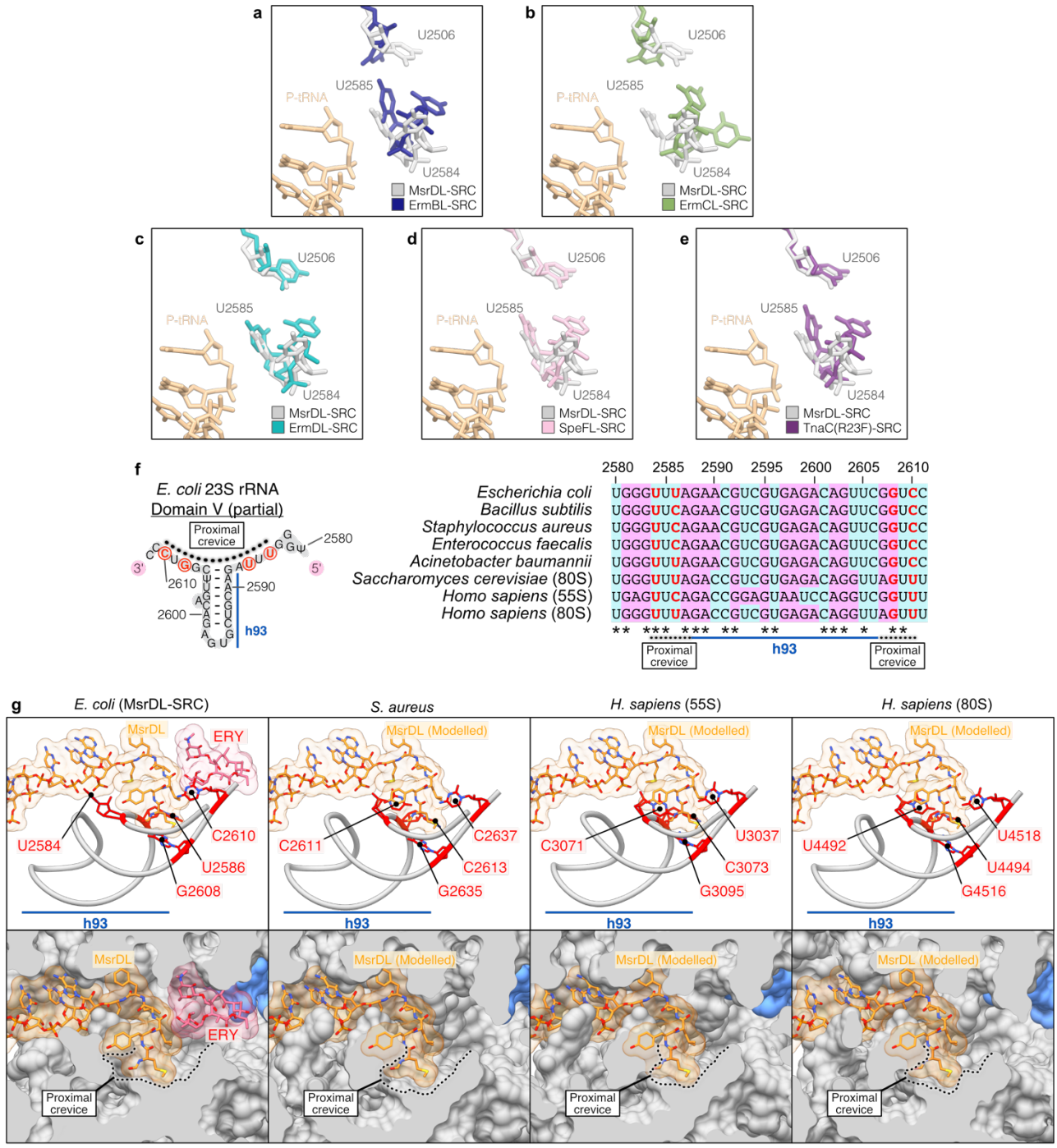
Supplementary figure 3. Biochemical characterization of MsrDL mode of action. (a) Schematic of the matrix TP_msrDL$_{WT}$ (Supplementary Table 5) used to generate synthetic mRNAs for *in vitro* experiments (RBS, ribosome binding site; NV1, annealing site for CY5-labelled primer). (b and c) Uncropped toe-printing gels presented in Fig. 3a and 3c. Formation of MsrDL-SRC would occlude formation of the Rho-independent transcription terminator (shown in light blue).

SUPPLEMENTARY FIGURE 4



Supplementary figure 4. Generation of the cryo-EM sample, data processing and model building. (a) Workflow for the generation of cryo-EM sample. See Methods for details. (b) Cryo-EM data processing workflow as described in the Methods. EMD accession numbers are indicated for each map. Maps obtained after multibody refinement are shown as transverse sections and colored according to local resolution. (c) Fourier Shell Correlation (FSC) curves of the 70S ribosome and the maps obtained after multibody refinement. (d) Transverse section of the composite map obtained after multibody refinement and isolated densities for MsrDL-tRNA and ERY, colored according to local resolution. The corresponding atomic reconstruction of the nascent chain is shown beside. (e) Details of the mRNA codon-tRNA anticodon interactions and corresponding densities for each nucleotide. (f) Details of clearly identifiable post-transcriptional modifications and corresponding densities.

SUPPLEMENTARY FIGURE 5



Supplementary figure 5. MsrDL engages within a universally conserved crevice at the NPET entrance. (a to e) Comparison of the conformation of 23S rRNA bases U2506, U2584 and U2585 for MsrDL-, ErmBL-, ErmCL-, ErmDL-, SpeFL- and TnaC(R23F)-stalled ribosomes structures [15–19](respectively PDB: 5JTE, 3J7Z, 7NSO, 6TC3, 7O1A). Structures were aligned based on domain V of 23S rRNA. (f) Sequence conservation of the proximal crevice between far-related species. The diagram shows a part of 23S rRNA domain V of *E. coli* and the location of proximal crevice at the base of h93. Large subunit rRNAs were aligned using Clustal W [5]

and visualized with Jalview [14] (purines, purple; pyrimidines, teal). Bases delimitating the proximal crevice (U2584, U2586, G2608 and C2610) are highlighted in red. Nucleotides numbering is relative to *E. coli* 23S rRNA sequence. (g) Structural conservation of the proximal crevice between far-related species. Top, cartoon representation of h93 and proximal crevice in *E. coli*, *S. aureus*, *H. sapiens* 55S mitoribosome and 80S cytosolic ribosome [20–22] (respectively PDB: 6YEF, 7A5F, 6OLI). Bases delimitating the proximal crevice are highlighted in red and numbered relative to the considered specie. Bottom, sagittal cut of ribosome tunnel shown on top panels depicted as surface. Ribosomal protein uL4 is shown in pale blue. Structures were aligned based on domain V of 23S rRNA.

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