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Supporting Information

Strengthening biofilms with selective metal ions

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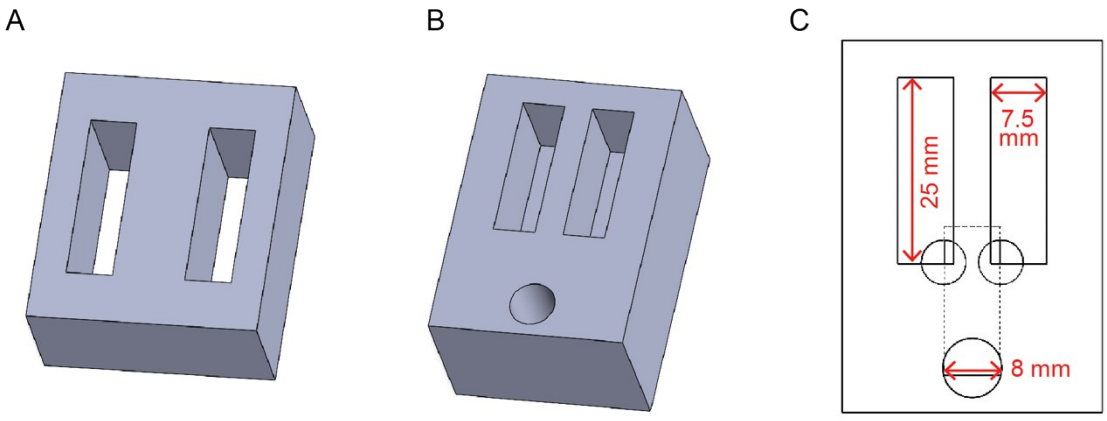

Figure S1. 3D renderings of pellicle culture molds used to generate rectangular biofilm strips for mechanical testing. Both molds have two 7.5 mm × 25 mm rectangular slits that define the sample culture regions. (A) Mold design which creates a pellicle within the mold region as well as throughout the cube culture container. Containers are filled with 35 mL of media, and pellicles form both inside the slits and across the liquid surface covering the culture container; only the in-mold pellicles are used, while those forming externally are sacrificed. Pellicles are floated from the mold by adding fluid to the sacrificed region to apply upward pressure beneath the culture slits. (B) Alternative mold design with an integrated media reservoir and a central media addition hole (shown in Movie S1). This version is filled with 7.5 mL of media directly into the mold. For testing, the pellicle that forms over the addition hole is first removed, and fresh media is added through the opening to float the biofilm strips from below using the same fluid pressure approach as in (A). (C) Internal view of the mold shown in (B), showing the fluid channel geometry and media addition port. Both culture configurations

were used in this study, with the setup in panel A employed for experiments involving metal ions to minimize potential carryover effects between treatments.

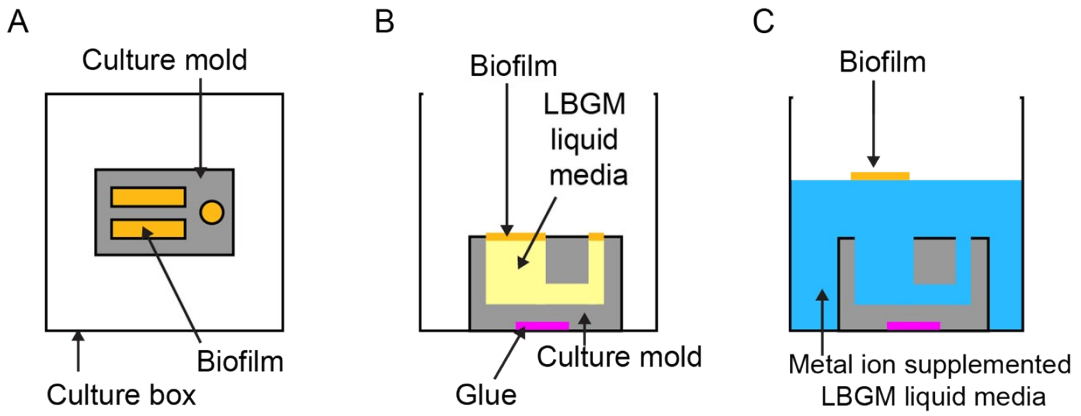

Figure S2. Schematic of pellicle floating and exposure procedure. (A) Top-down view of a 3D-printed culture mold placed inside a Magenta Plant Culture box, showing fully formed B. subtilis pellicles within the mold cavities. (B) Side view (cross-section) of the culture mold during growth, with LBGM (LB-glycerol-manganese) media beneath the pellicle at the air–liquid interface. (C) Side view (cross-section) after addition of liquid to float the pellicle from the mold. At this stage, pellicles are exposed for 1 h to metal ion-supplemented media, pH-adjusted LBGM, or wash solutions prior to mechanical testing.

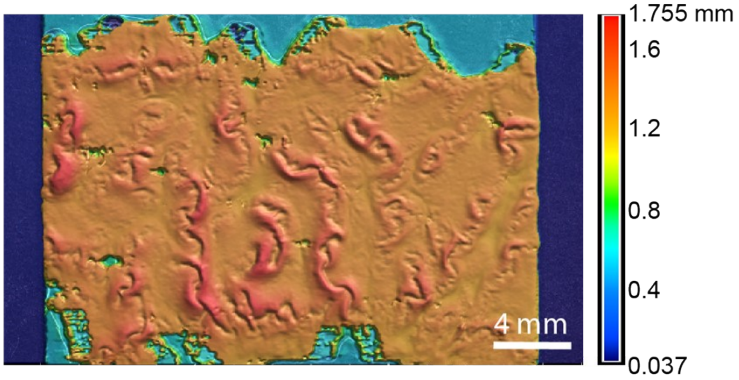

Figure S3. Representative optical profilometry scan of a *B. subtilis* pellicle atop a glass slide. Average thickness was determined to be 356 ± 53 μm for this sample.

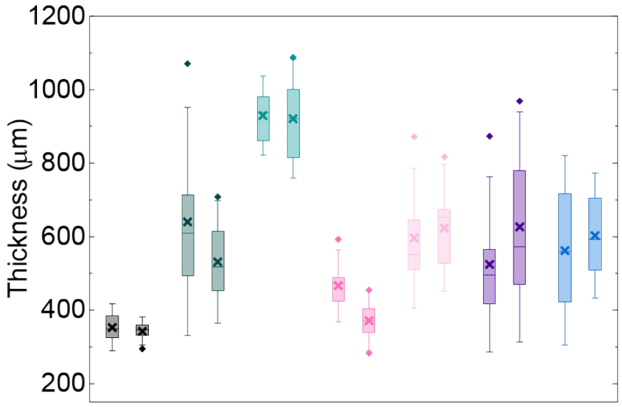

Figure S4. Comparison of the thickness of the two pellicle strips generated from the same culture mold (represented by two bars with the same color). Boxes represent the range from 25%-75%, whiskers represent the standard deviation, horizontal lines represent the median, and × symbols represent the mean.

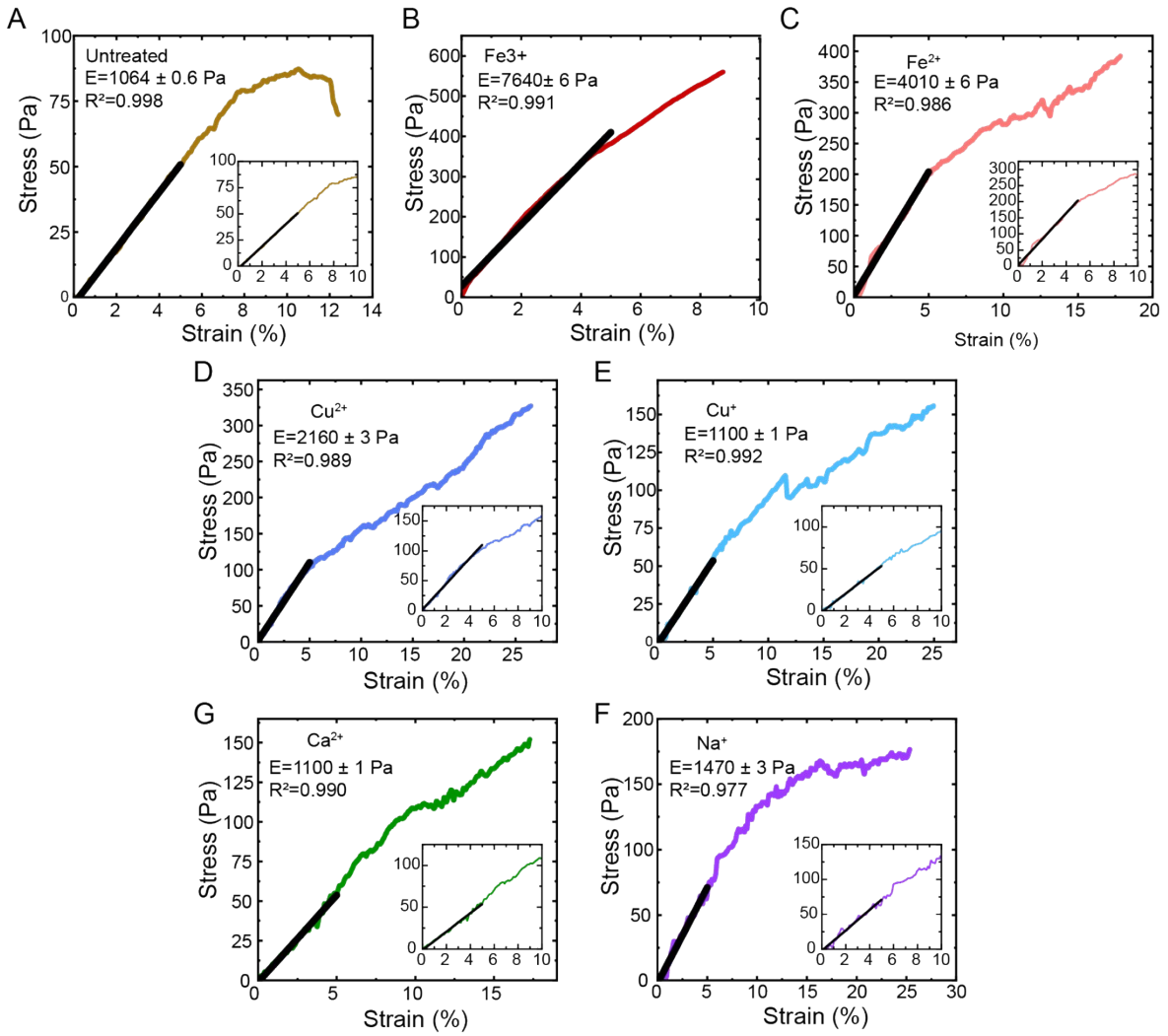

Figure S5. Low-strain region of representative stress-strain curves for metal ion treatments. The black line overlaid on each curve is the linear fit applied at fixed strain range of 0-5% to determine the low strain elastic modulus (*E*). For each curve, the coefficient of determination (R^2) of the linear fit and the *E,* calculated from the slope of the fit and the standard error (slope × 100) are reported.

Table S1. Average low strain elastic modulus values determined from linear fits of stress-strain data over the 0.5-5% strain and 0-5% strain ranges.

Treatment	Modulus, 0.5-5% strain (Pa)	Modulus, 0-5% strain (Pa)
Untreated	845±316	837±318
Fe^{3+}	8440±2330	8820±2330
Fe^{2+}	27300±947	2870±1010
Cu^{2+}	2450±1030	2500±1040
Cu^{+}	1220±347	1190±333
Ca^{2+}	1100±351	1080±336
Na^{+}	1390±102	1370±99.8

Table S2. Average low strain elastic modulus values determined from linear fits of stress-strain data over the 0-5% strain and 0-10% strain ranges.

Treatment	Modulus, 0-5% strain (Pa)	Modulus, 0-10% strain (Pa)
Untreated	837±318	767±264
Fe^{3+}	8820±2330	5080±2550
Fe^{2+}	2870±1010	1890±844
Cu^{2+}	2500±1040	2000±1030
Cu^{+}	1190±333	1050±248
Ca^{2+}	1080±336	846±194
Na^{+}	1370±99.8	1190±145

Table S3. Average modulus (Pa), failure strain (%), maximum stress at failure (Pa), and yield stress (Pa) for untreated B. subtilis pellicles as well as those treated with 50 mM $FeCl_3$, $CuSO_4$, $CaCl_2$, or NaCl for 1 hour. Data is filtered and presented as an average (if n ≥2) standard deviation (if n≥3) for the presence of a pre-existing crack (if n=1, recorded values represent a single data point). Cracks often initiated during pellicle removal and transfer from culture molds.

Treatment	Pre-existing crack? (Y/N)	# samples (n)	Low strain modulus (Pa)	Failure strain (%)	Max stress (Pa)	Yield Stress (Pa)
Untreated	N	6	751±244	28.4±15.1	124±67.1	83.3±47.1
	Y	1	1350	10.4	97.5	97.5
Fe^{3+}	N	6	9400±1900	9.76±4.53	666±225	605±226
	Y	1	5290	10.7	305	258
Fe^{2+}	N	5	2870±1010	20.5±13.0	267±136	185±80.6
	Y	0	N/A	N/A	N/A	N/A
Cu^{2+}	N	5	2660±1220	18.8±8.02	339±213	271±123
	Y	2	2080	28.7	312	228
Cu^{+}	N	4	1170±87.2	36.9±17.7	149±43.2	112±42.0
	Y	2	1210	17.5	164	136
Ca^{2+}	N	5	1030±342	21.3±6.70	130. ±33.7	116±34.2
	Y	4	1130±360	16.2±5.16	108±37.0	100. ±34.9
Na^{+}	N	6	1370±99.8	17.8±8.08	142±29.2	130. ±27.0
	Y	0	N/A	N/A	N/A	N/A

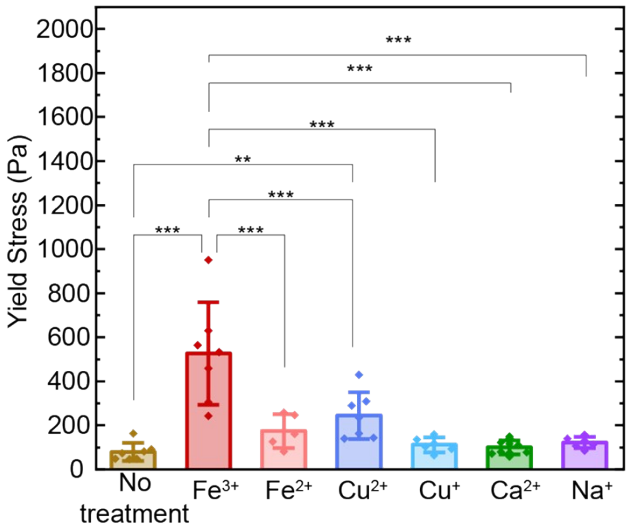

Figure S6. Comparison of average yield stress for untreated pellicles (n=7) as well as those incubated in media supplemented with 50 mM $FeCl_3$ (n=7), $FeCl_2$ (n=5), $CuSO_4$ (n=7), $CaCl_2$ (n=10), CuCl (n=6), or NaCl (n=6) for one hour. Yield stress was identified qualitatively as the point where the tangent slope of the stress-strain curve decreased substantially relative to the initial linear regime. If no clear yield point is identifiable, maximum stress is used for that sample. Data are represented as mean ± standard deviation (SD). Statistical significance is determined using a one-way Analysis of Variance (ANOVA) with a Fisher's Least Significant Difference (LSD) post hoc test. $p<0.05$, $p<0.01$, and $p<0.001$ is reported with a *, **, and *** respectively.

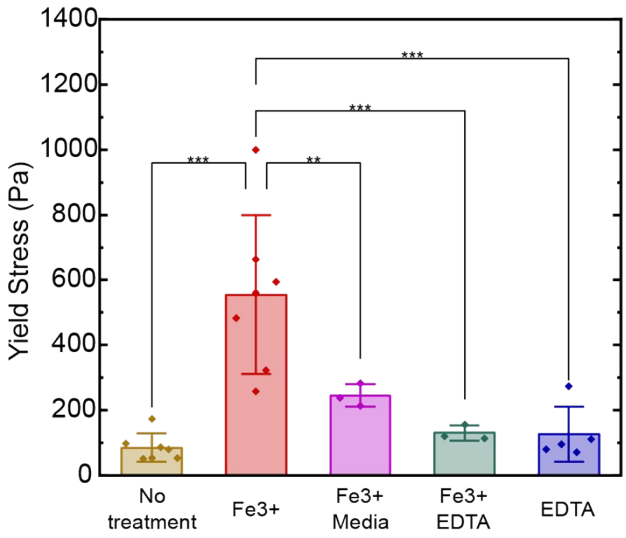

Figure S7. Comparison of average yield stress across treatment groups (untreated (n=7), Fe^{3+} (n=7), EDTA wash (n=3), LBGM wash (n=3), EDTA only (no Fe^{3+} pre-treatment, n=5)). Yield stress was identified qualitatively as the point where the tangent slope of the stress-strain curve decreased substantially relative to the initial linear regime. If no clear yield point

is identifiable, maximum stress is used for that sample. Data are represented as mean ± SD (n³3). Statistical significance is determined using a one-way ANOVA with a Fisher's LSD post hoc test. $p<0.05$, $p<0.01$, and $p<0.001$ is reported with a *, **, and *** respectively.

Table S4. pH of media and all treatments

Treatment	pH
LBGM Media	7.0
50 mM $FeCl_3$ in LBGM	2.1
50 mM FeCl2 in LBGM	5.6
50 mM $CuSO_4$ in LBGM	3.5
50 mM CuCl in LBGM	5.9
50 mM $CaCl_2$ in LBGM	6.5
50 mM NaCl in LBGM	6.9
25 mM EDTA	5.1

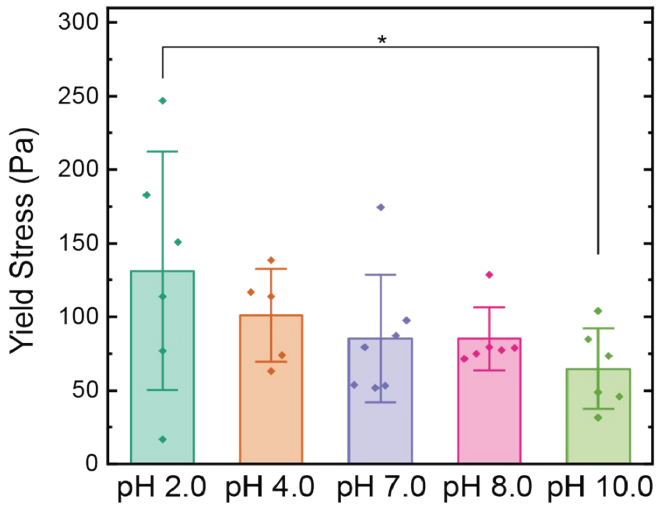

Figure S8. Comparison of average yield stress for *B. subtilis* pellicles treated with LB-glycerol-manganese (LBGM) media adjusted to pH 2.0 (n=5), 4.0 (n=5), 8.0 (n=6), and 10.0 (n=6). Yield stress was identified qualitatively as the point where the tangent slope of the stress-strain curve decreased substantially relative to the initial linear regime. If no clear yield point is identifiable, maximum stress is used for that sample. Data are represented as mean ± standard deviation ($n \geq 3$). Statistical significance was determined using a one-way ANOVA with a Fisher's Least Significant Difference (LSD) post hoc test. $p<0.05$, $p<0.01$, and $p<0.001$ is reported with a *, **, and *** respectively.

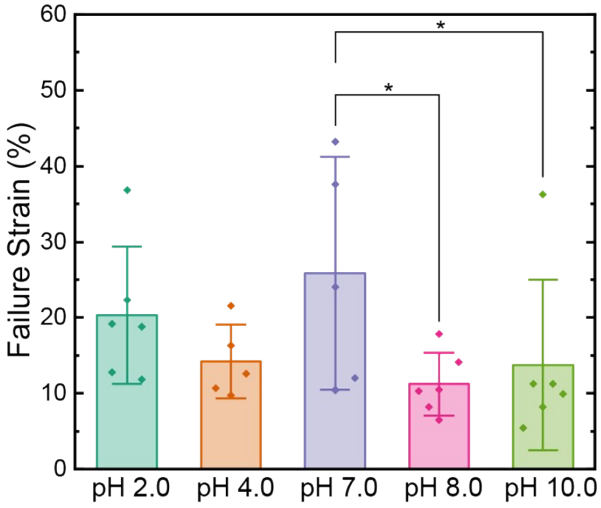

Figure S9. Comparison of average failure strain for *B. subtilis* pellicles treated with LB-glycerol-manganese (LBGM) media adjusted to pH 2.0 (n=5), 4.0 (n=5), 8.0 (n=6), and 10.0 (n=6). Data are represented as mean ± standard deviation ($n \geq 3$). Statistical significance was determined using a one-way ANOVA with a Fisher's Least Significant Difference (LSD) post hoc test. $p<0.05$, $p<0.01$, and $p<0.001$ is reported with a *, **, and *** respectively.