Supplemental Figures:

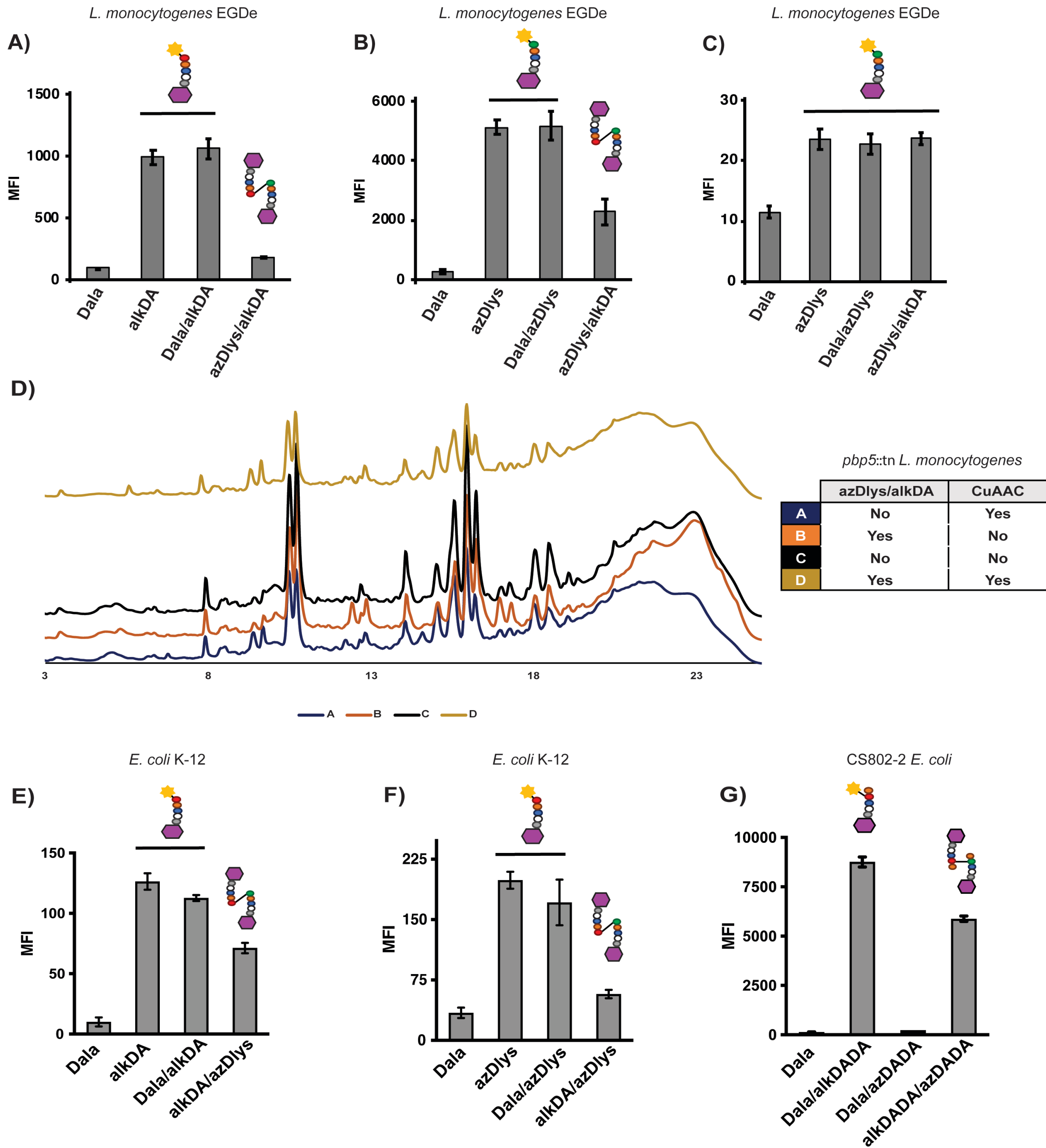


Figure S1. Indirect evidence for synthetic cross-links in *Listeria monocytogenes* and *Escherichia coli*, related to Figures 1 and 2. (A-C) and (E-F) loss of fluorescence from monopeptide D-amino acid labeling followed by CuAAC. Wild-type *L. monocytogenes* EGDe (A-C) and *E. coli* K-12 (E-F) were incubated in the presence of the indicated D-amino acids, washed, and subjected to CuAAC with either azido- (A, E) or alkynyl-fluorophore (B, F) or to SPAAC with DBCO-CR110 (C). (D) *L. monocytogenes* peptidoglycan analysis. The *pbp5*::tn mutant was incubated or not with

a mixture of alkyne-D-alanine (alkDA) and azido-D-lysine (azDlys), washed, and subjected to CuAAC with BTTP ligand. Peptidoglycan was extracted, digested with mutanolysin and lysozyme, and separated by ultra-performance liquid chromatography (UPLC). We were unable to detect differences between the chromatograms that were specific to both alkDA/azDlys and CuAAC treatments, presumably because of the pre-existing complexity of the Gram-positive peptidoglycan. Experiment was performed twice. (G) Loss of fluorescence from dipeptide D-amino acid labeling followed by CuAAC. CS802-2 *E. coli* were incubated in the presence of N-terminally-modified D-amino acid dipeptides azido-D-alanine-D-alanine (azDADA) or alkynyl- D-alanine-D-alanine (alkDADA), washed, and subjected to CuAAC with BTTP ligand and picolyl azide AF488.For (A-C) and (E-G), fluorescence was quantitated by flow cytometry and data are representative of 2-6 biological replicates performed in technical triplicate. MFI, mean fluorescence intensity. Error bars, +/- standard deviation of technical replicates.

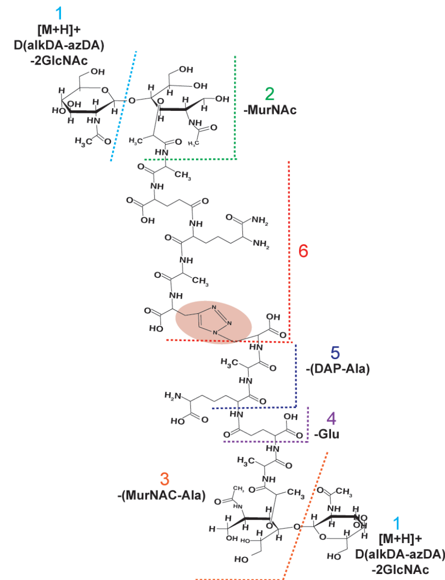
A)

Ion	Fragment lost	[H+M] + Observed	[H+M] + Calculated
Parental Ion	-	2090.878	2090.898
1	GlcNAc	1887.817	1887.811
2	GlcNAc, GlcNAc	1684.633	1684.724
3	GlcNAc, GlcNAc, MurNAc	1407.681	1407.608
4	GlcNAc, GlcNAc, MurNAc, MurNAc, Ala	1059.423	1059.455
5	GlcNAc, GlcNAc, MurNAc, MurNAc, Ala, Glu	930.38	930.412
6	GlcNAc, GlcNAc, MurNAc, MurNAc, Ala, Glu, DAP, Ala	687.279	687.29

B)

	alkDa	azDA	
	Dala/alkDA -CuAAC	Dala/azDA -CuAAC	Dala/azDA +CuAAC
% PG-Modification	6.205 ± 0.81	10.615 ± 0.01	11.695 ± 1.08
% Cross-linkage	53.8575 ± 1.15	46.84 ± 7.707	48.5175 ± 5.53

D)



C)

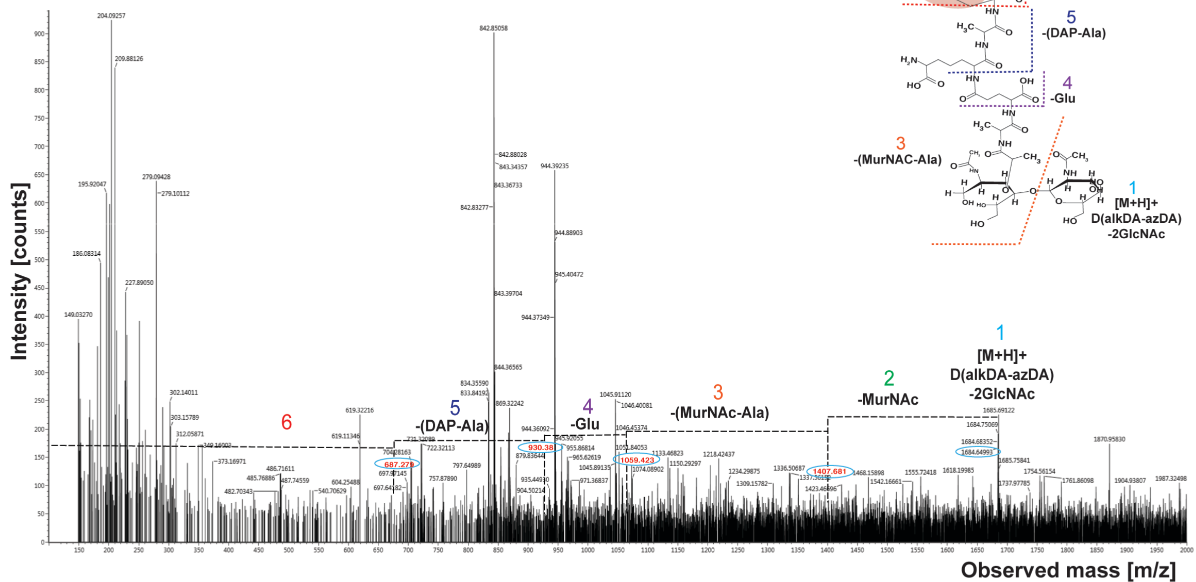


Figure S2. Biochemical characterization of synthetic cross-links in *E. coli*, related to Figure 2. (A) MS/MS fragmentation of 5-5 triazole dimer. (B) Quantification of muropeptides from CS802-2 *E. coli* treated with Dala/alkDA or Dala/azDA and subjected or not to CuAAC. (C-D) Chemical structure and MS/MS profile of 5-5 triazole dimer. Data are from two biological replicates.

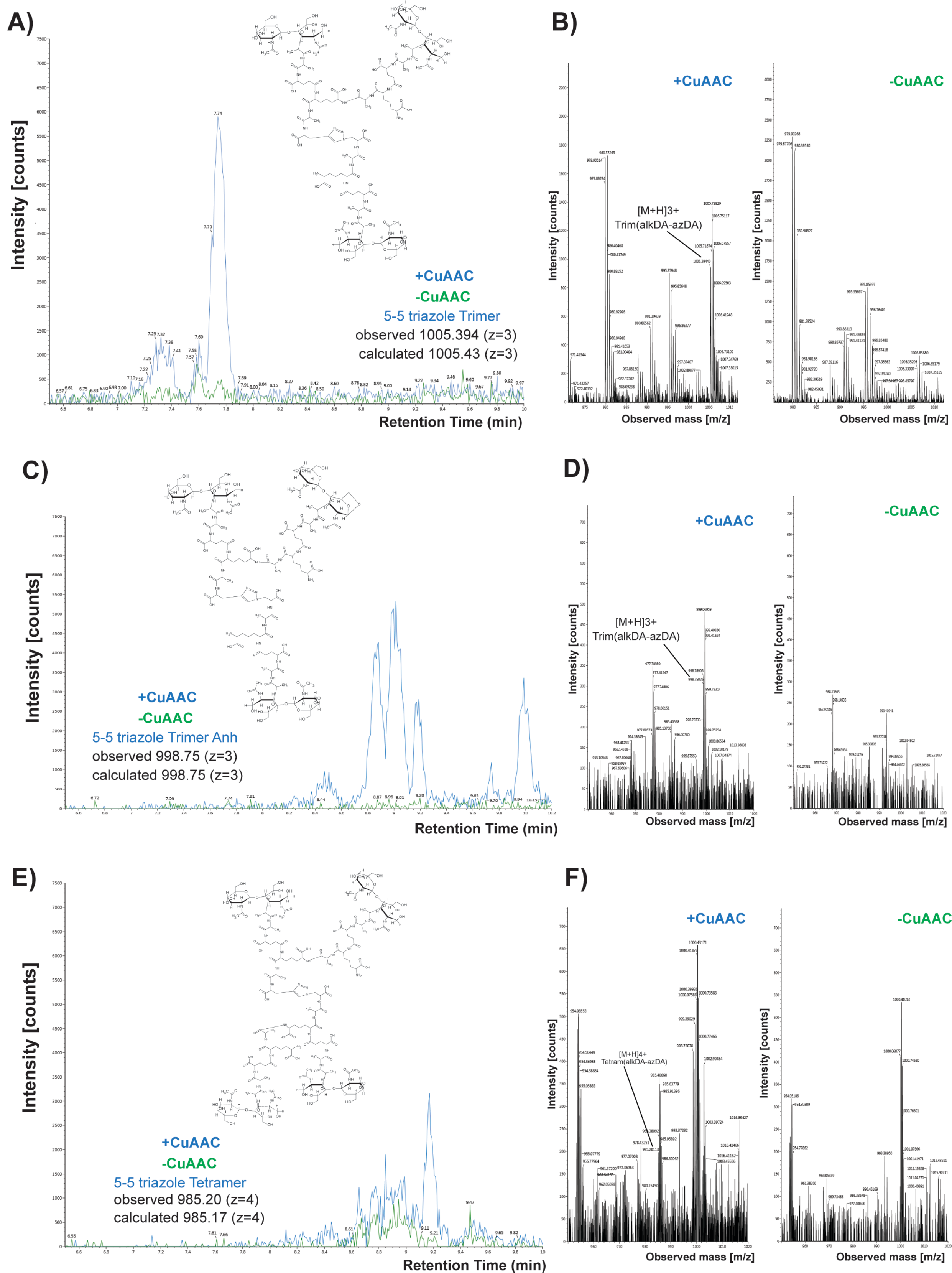


Figure S3. Ion detection and MS profiles of 5-5 triazole-linked trimers, related to

Figure 2. Ion detection (left) and MS profiles (right) for 5-5 triazole-linked trimer (A,B), trimer anh (C,D), and tetramer (E,F) from alkDA/azDA-labeled bacteria +/- CuAAC.

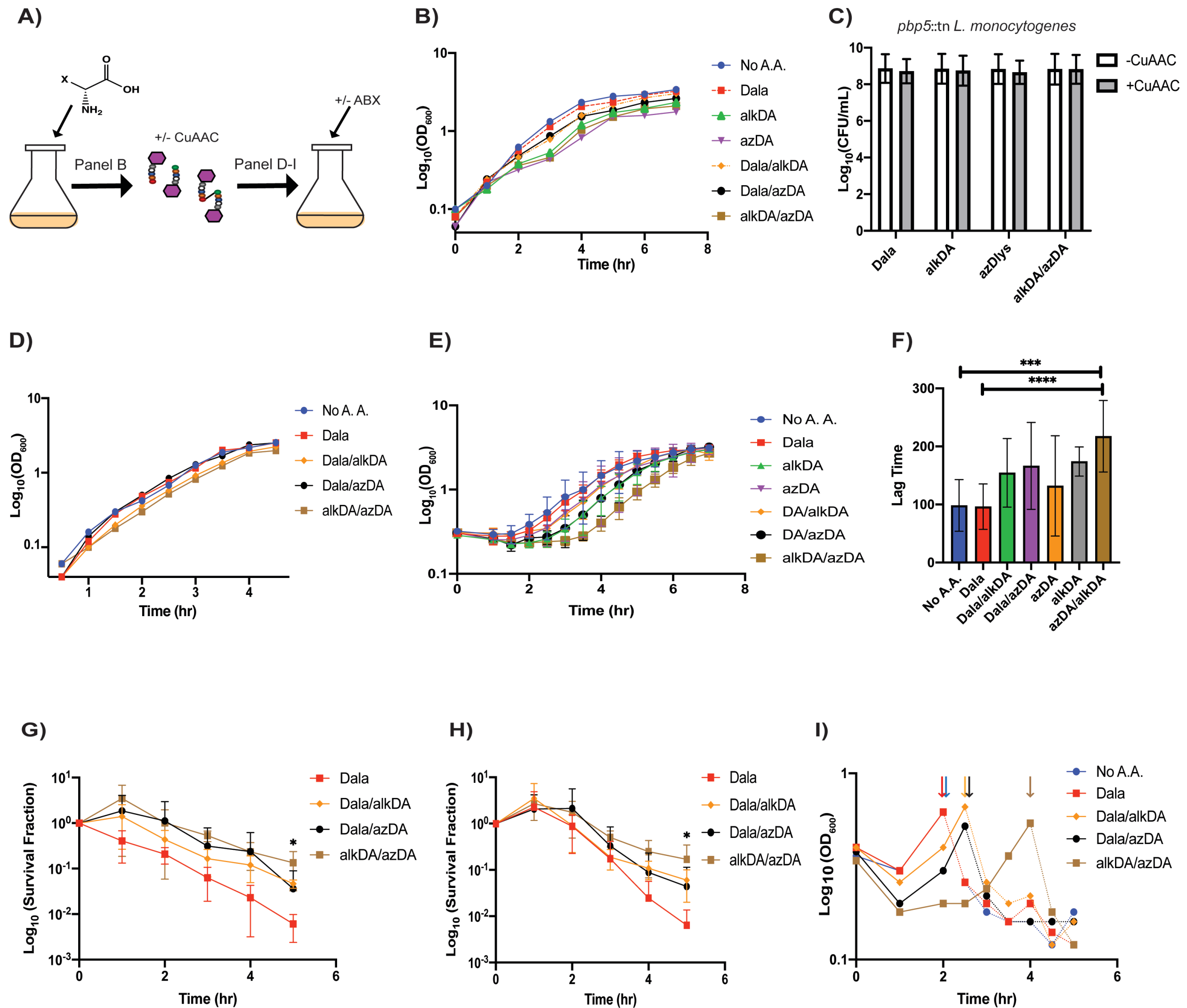


Figure S4. Impacts of D-amino acids and/or CuAAC on bacterial growth, viability and β-lactam susceptibility, related to Figure 3. (A) Experimental strategy for panels (B), (D-I), and Figure 3. ABX, antibiotics. (B) Growth of CS802-2 *E. coli* in the presence of the indicated D-amino acids pre-CuAAC. While Dala alone had no effect on growth, we observed a small but reproducible lag between 2 and 3 hours of incubation when CS802-2 *E. coli* was incubated at higher concentrations of alkDA and/or azDA. However, the growth rates stabilized and were similar between the strains after 4 to 5 hours of incubation. (C) *pbp5*::tn *L. monocytogenes* was incubated in the presence of the indicated D-amino acids, washed, and subjected or not to BTTP-liganded CuAAC. Serial dilutions were plated at the indicated time points and assessed the next day for colony-forming units (CFUs). (D-F) CS802-2 *E. coli* were labeled with the indicated combinations of D-amino acids overnight prior to BTTP-liganded CuAAC. Growth of D-amino acid-treated bacteria post-CuAAC (E) or mock treatment (D). (F) Quantitation of lag time from (E). (G-H) CS802-2 *E. coli* were treated with indicated combinations of D-amino acids, subjected to CuAAC, and immediately transferred to growth medium with (G) ampicillin or (H) carbenicillin. Serial dilutions were plated at the indicated time points

and assessed for colony-forming units (CFUs). The experiment in (I) was performed similarly except that ampicillin was added *after* bacteria were out of lag phase (arrow), at OD_{600} 0.5-0.6. Growth prior to ampicillin, solid lines; lysis in the presence of ampicillin, dashed lines. Error bars, +/- standard deviation. For (B), (D) and (I) data are representative of 2-5 biological replicates. For (C), (E-H) data are average of 3-12 biological replicates performed in triplicate. Statistical significance assessed by one- (F) or two-way (G-H) ANOVA with Tukey's multiple comparison test. *, $p<0.05$; ***, $p<0.0005$; ****, $p<0.00005$. For (G-H), only the differences between strains treated with D-alanine and the combination of alkDa/azDA were significant.