Supporting Information

A Fluorescent Transport Assay Enables Studying AmpG Permeases Involved in Peptidoglycan Recycling and Antibiotic Resistance.

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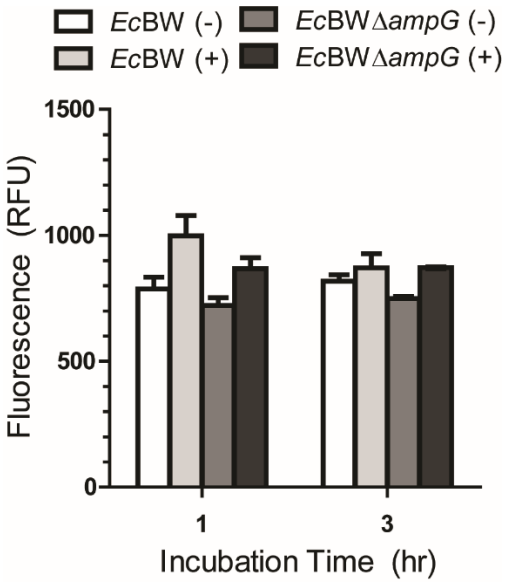
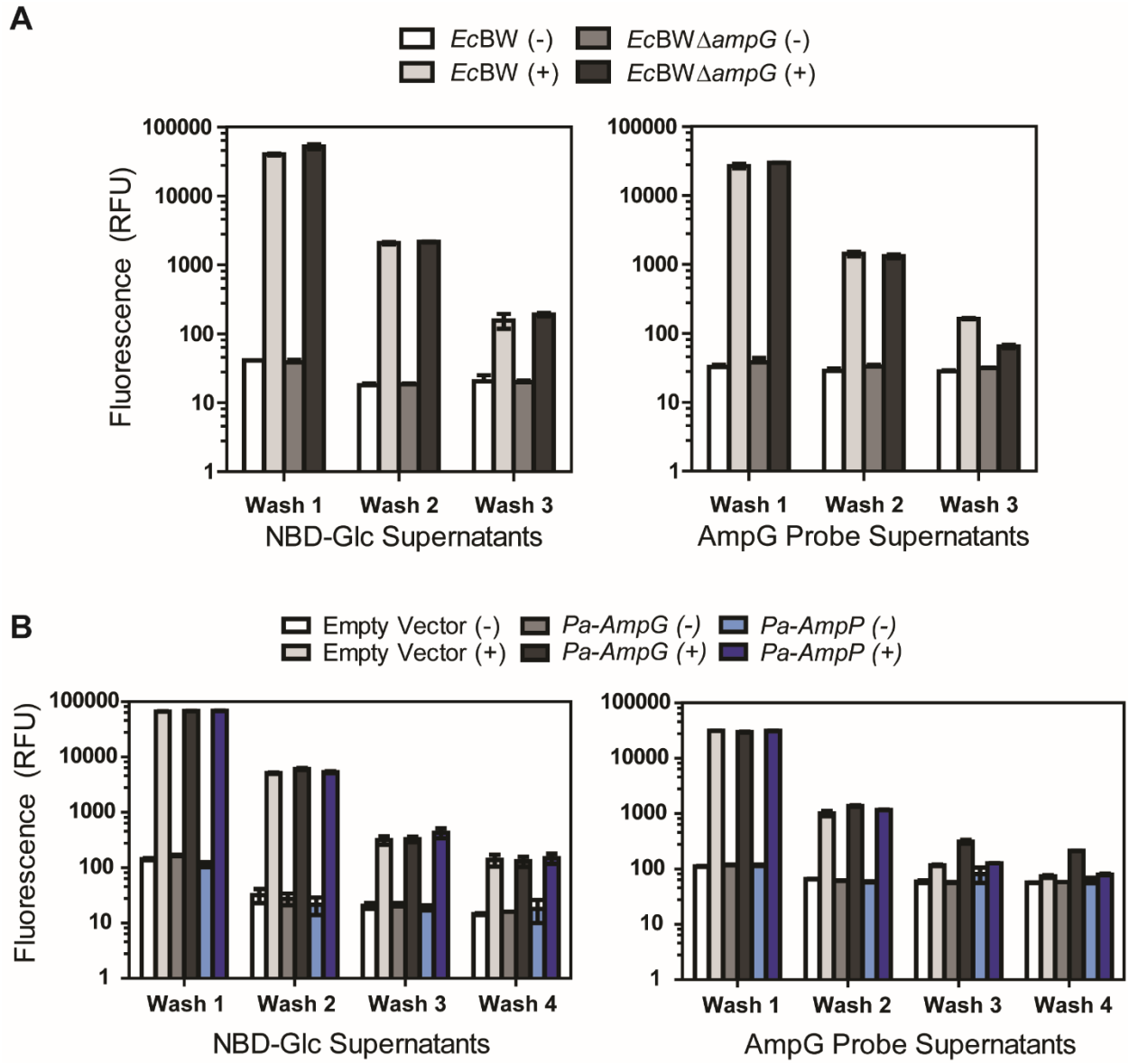
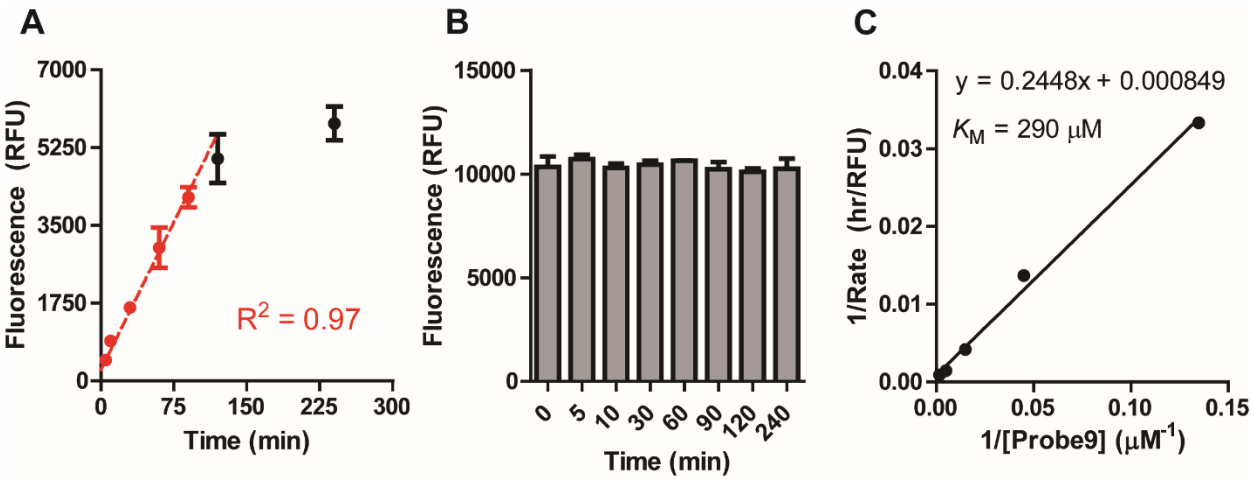
Supporting Figures



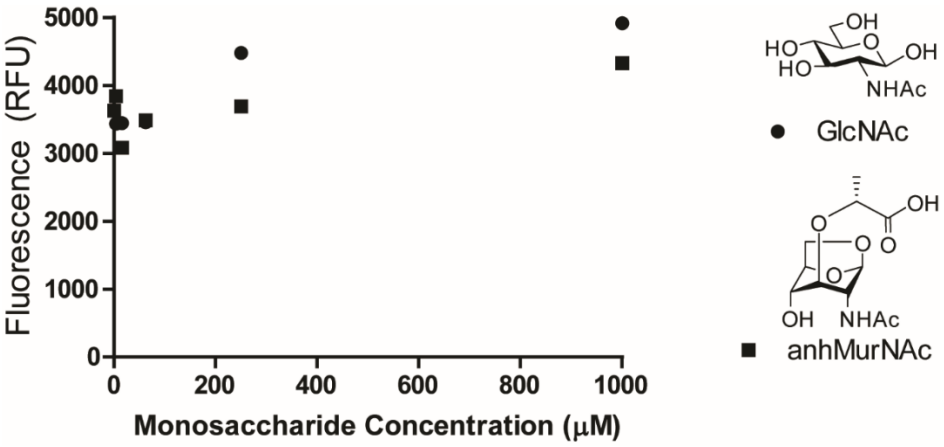
Supplementary Figure 1. Neither whole cell *Ec*BW nor *Ec*BWΔ*ampG* are capable of internalizing probe 9, regardless of incubation time. Fluorescence observed for native *Ec*BW and *Ec*BWΔ*ampG* bacteria after incubation in the presence (+) or absence (-) of probe **9**. Error bars represent the standard error of the mean.



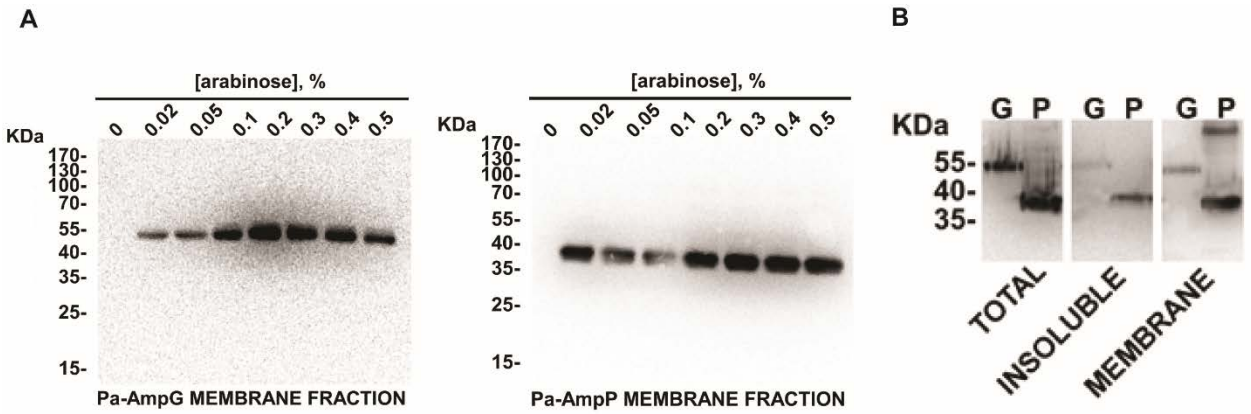
Supplementary Figure 2. Three washes were sufficient to remove the vast majority of excess NBD-Glc or probe 9. (**A**) Supernatant fluorescence after one to three washes of *Ec*BW or *Ec*BW*ΔampG* spheroplasts which were incubated with (+) or without (-) NBD-Glc or probe **9**. (**B**) Supernatant fluorescence after one to four washes of *Ec*MG*ΔampG* spheroplasts bearing the empty vector, or a plasmid expressing V5 His_6 tagged *Pa*-AmpG or *Pa*-AmpP, which were incubated with (+) or without (-) NBD-Glc or probe **9**. Error bars represent the standard error of the mean. Data plotted on a logarithmic y-axis.



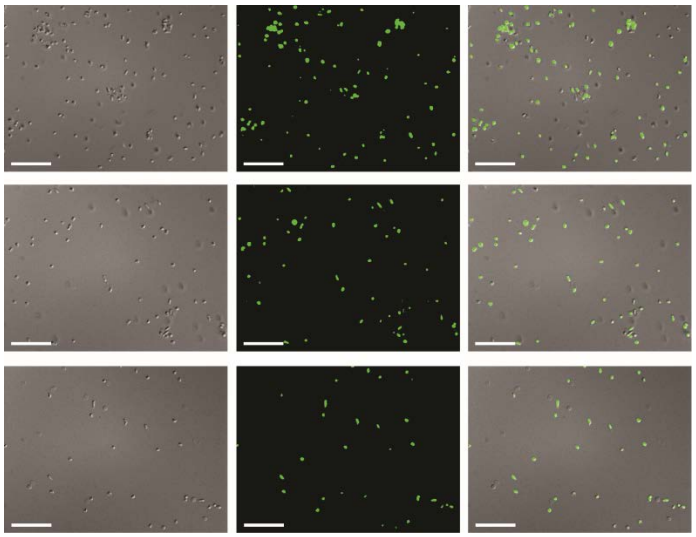
Supplementary Figure 3. *Ec*BW spheroplasts exhibit linearity in the initial uptake of probe 9, the amount of extravesicular probe remains constant, and a Lineweaver-Burk plot of the data in Figure 3 gives a K_M value similar to that found by non-linear regression. **A**: Time course for the uptake of probe **9** into *Ec*BW spheroplasts. A line of best fit is indicated for the first five time points, up to and including 90 min, to indicate the approximately linear rate of probe internalization over this period. **B**: Supernatant fluorescence from time course remains constant, indicating that only a small amount of probe **9** is internalized. Error bars represent the standard error of the mean. **C**: The five highest concentrations from Figure 3 were plotted on reciprocal x- and y-axes, and the K_M was determined from the negative reciprocal of the x-intercept.



Supplementary 4. Monosaccharides GlcNAc and anhMurNAc have no impact on the AmpG-mediated transport of probe 9. Fluorescence observed for *Ec*BW spheroplasts after incubation with a range of GlcNAc or anhMurNAc concentrations in the presence of probe **9**. This experiment was carried out in parallel with the competitive assay which used GlcNAc-anhMurNAc, indicating the viability of the spheroplasts.

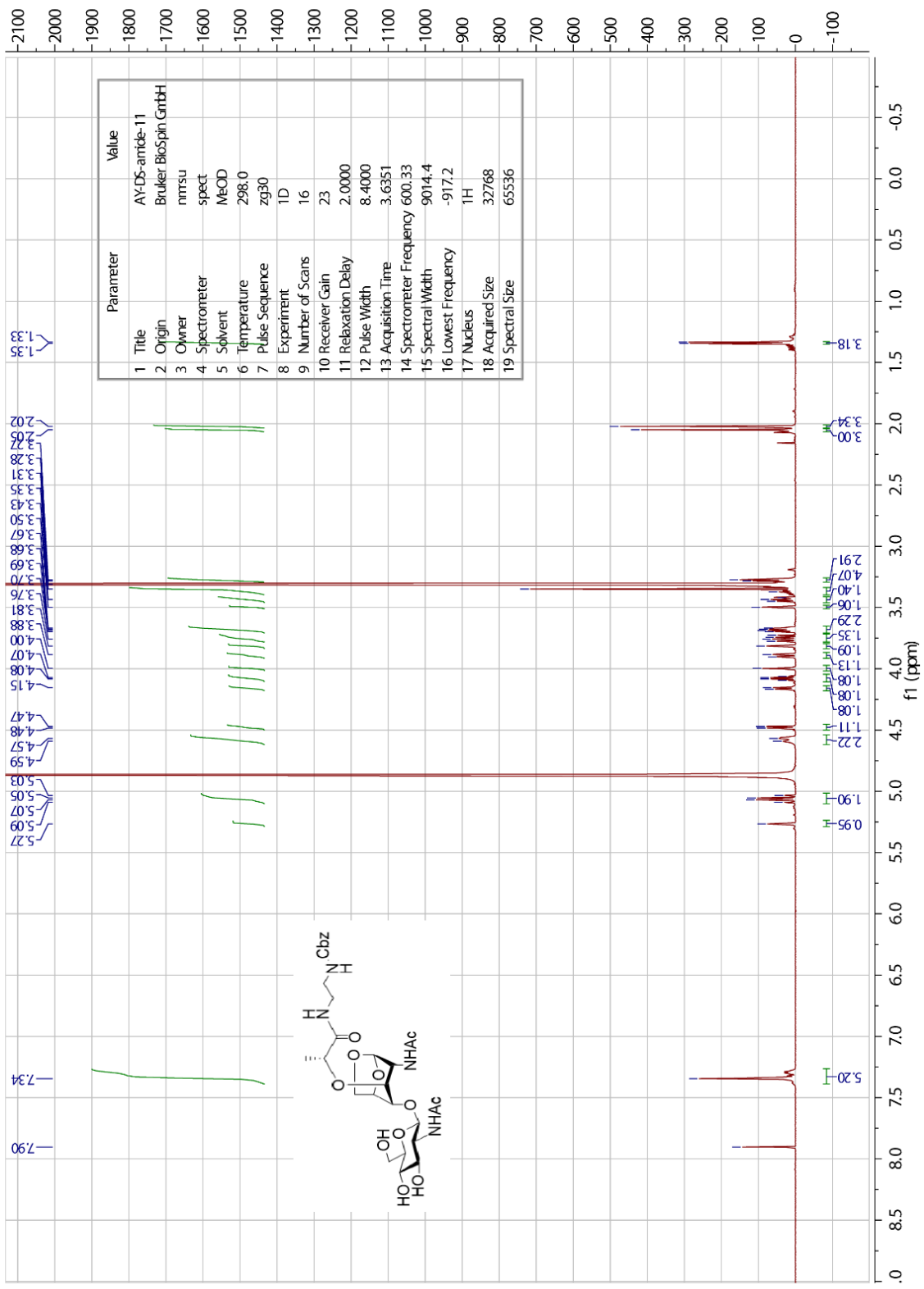


Supplementary Figure 5. V5 His_6 tagged *Pa*-AmpG and *Pa*-AmpP expression is saturated at 0.2% inducer, and both proteins are targeted to the membrane under spheroplasting conditions. (**A**) The expression of both *Pa*-AmpG and *Pa*-AmpP depends on the concentration of inducer (L-arabinose). It should be noted that when the cells are grown in LB medium, the expression levels of *Pa*-AmpG and *Pa*-AmpP become saturated at 0.2% arabinose as shown. We therefore chose 0.2% inducer in LB-grown cultures for the purposes of isolating spheroplasts. (**B**) Membrane fractionation of *Ec*MGΔ*ampG* spheroplasts expressing *Pa*-AmpG (G) or *Pa*-AmpP (P) indicates that the proteins are properly targeted to the inner membrane



Supplementary Figure 6. AmpG knockout spheroplasts (EcMGΔampG) which were transformed with an empty vector, Pa-AmpG, or Pa-AmpP, are all able to internalize NBD-Glc, indicating they are comparably viable. (**A**) DIC (left), fluorescence (center), and overlay (right) images of EcBWΔampG spheroplasts transformed with the empty vector (pBAD322-V5His-Km, top), or the V5His tagged proteins Pa-AmpG (pBAD-Pa-AmpG-V5His-Km, center) or Pa-AmpP (pBAD-Pa-AmpP-V5His-Km, bottom) after incubation with NBD-Glc. A 20 μm bar is included for scale. An excitation wavelength of 470 nm was used.

NMR Spectra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NHAc
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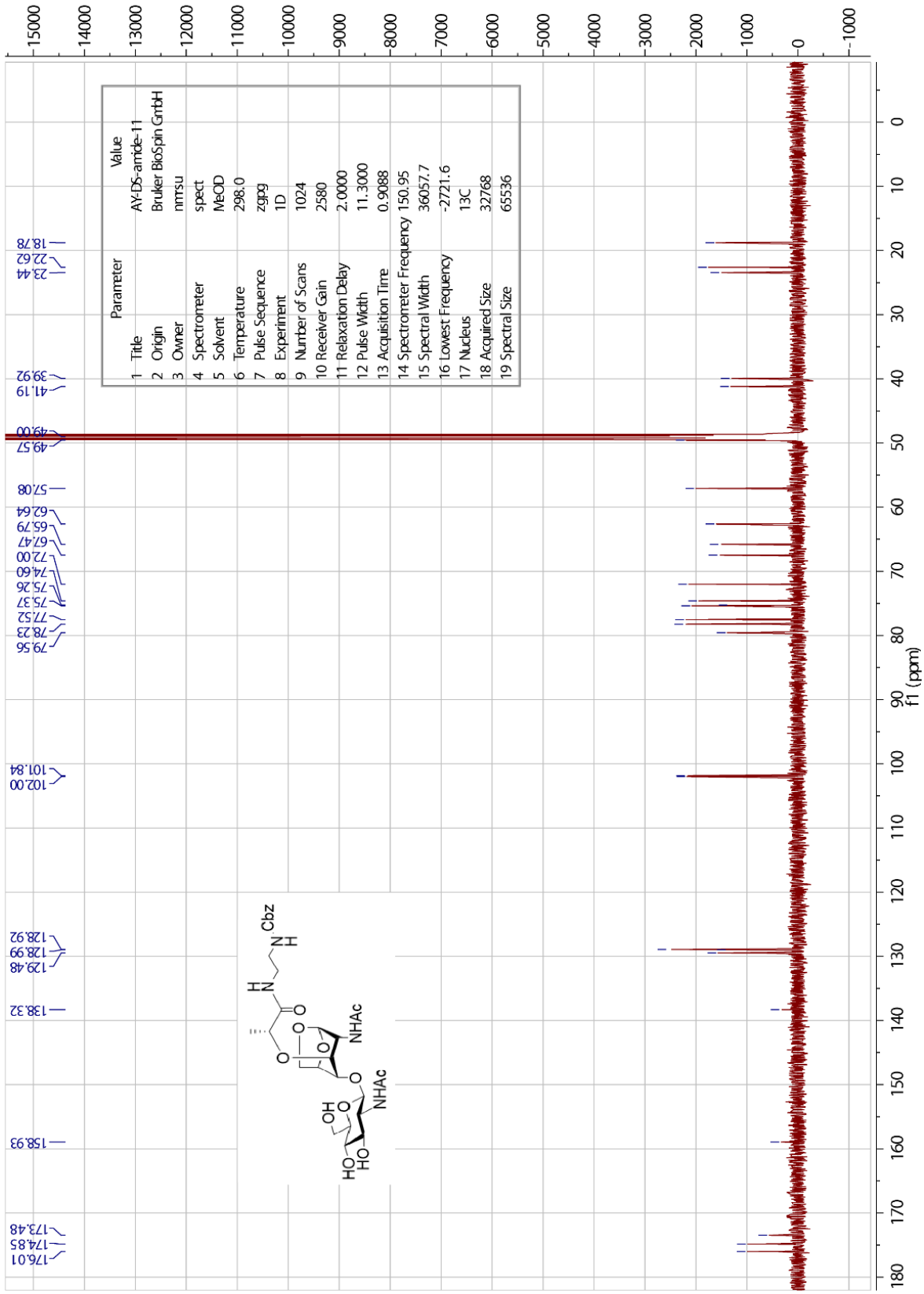
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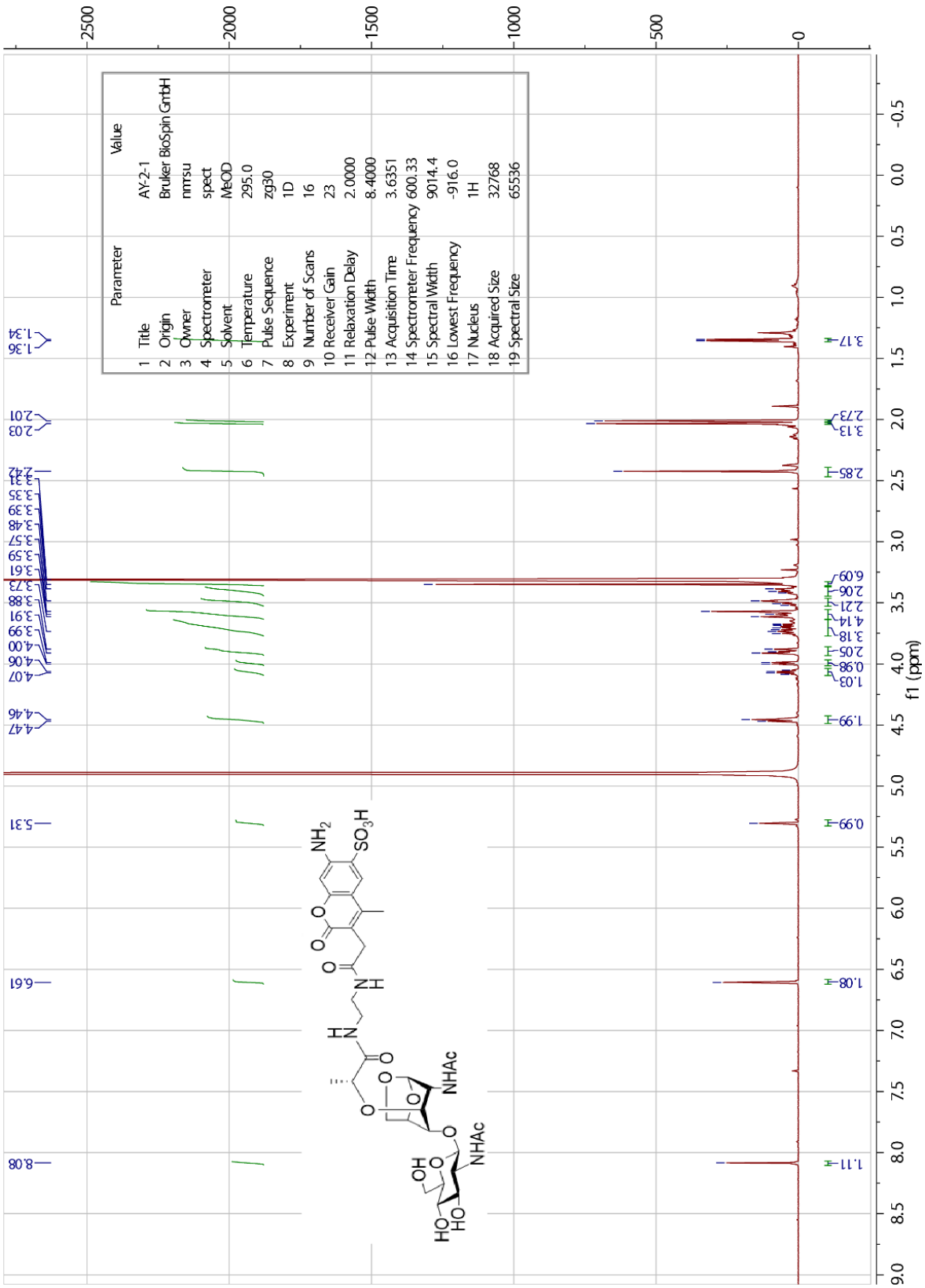
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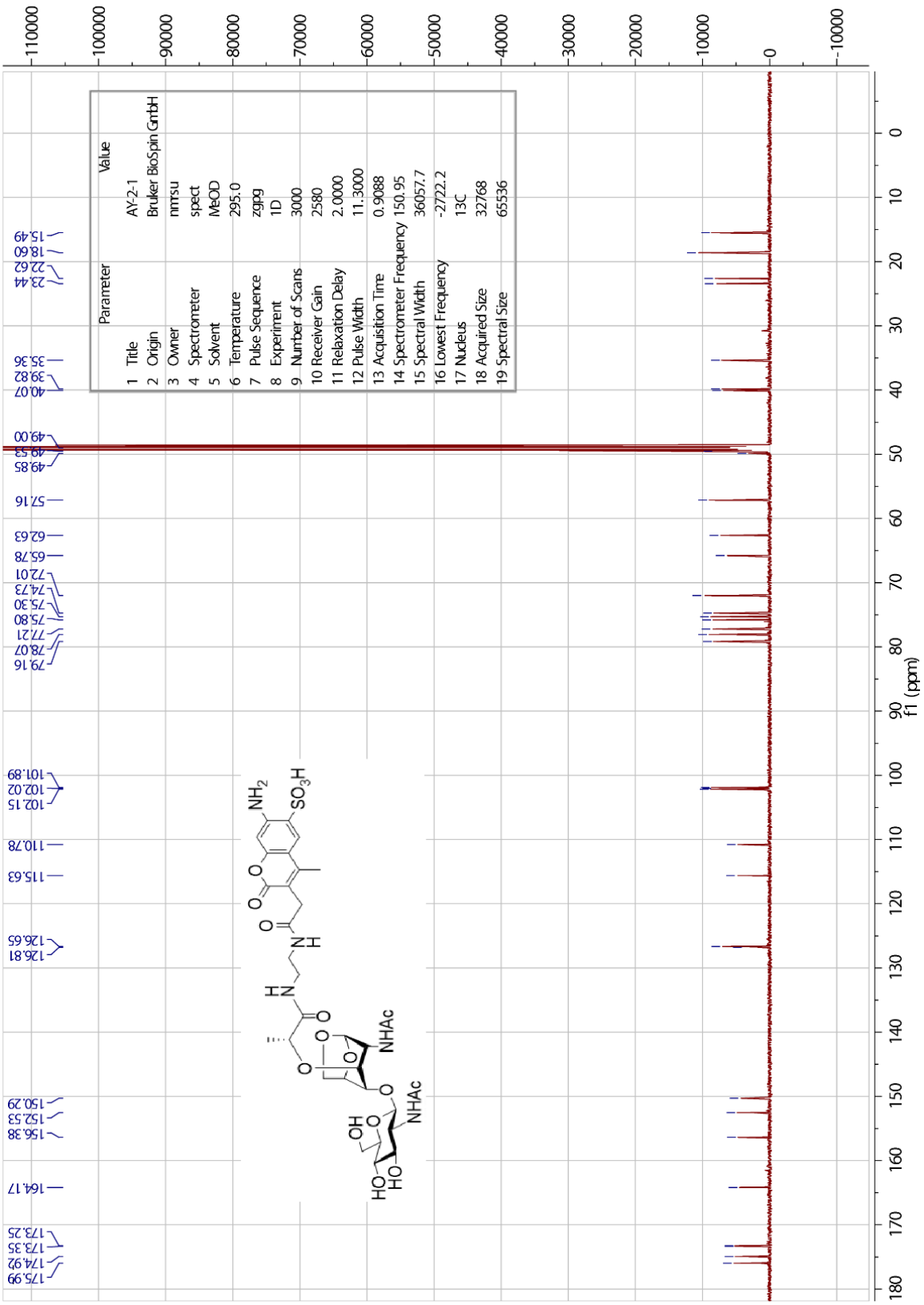
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101.89
102.02
102.15
110.78
115.63
126.65
126.81
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152.53
156.38
164.17
173.25
173.35
174.92
175.99
NH2
SO3H
NHAc
NHAc
f1 (ppm)

Supporting Methods

Characterization of Key Chemical Compounds. *Compound **8***. R_f 0.6 (6:3:1 EtOAc:MeOH:H2O); HRMS (ESI-TOF) m/z 655.2823 (655.2821 calcd for $C29H43N4O13^+$, $[M+H]^+$); ^{1}H-NMR (600 MHz, MeOD) δ 7.33 – 7.25 (m, 5H), 5.24 (s, 1H), 5.04 (q, J = 12.5, 2H), 4.54 (d, J = 5.4, 1H), 4.45 (d, J = 8.4, 1H), 4.14 (d, J = 7.5, 1H), 4.05 (q, J = 6.8, 1H), 3.97 (s, 1H), 3.87 (dd, J = 11.9, 1.9, 1H), 3.79 (s, 1H), 3.76 – 3.70 (m, 1H), 3.68 – 3.63 (m, 2H), 3.47 (s, 1H), 3.41 (dd, J = 10.5, 8.2, 1H), 3.37 – 3.33 (m, 1H), 3.32 (s, 1H), 3.31–3.30 (m, 1H), 3.25 (dd, J = 9.7, 3.8, 3H), 2.02 (s, 3H), 2.00 (s, 3H), 1.32 (d, J = 6.8, 3H); ^{13}C-NMR (151 MHz, MeOD) δ 176.05, 174.90, 173.52, 158.98, 138.35, 129.53, 129.04, 128.96, 102.05, 101.88, 79.61, 78.26, 77.58, 75.41, 75.32, 74.65, 72.07, 67.53, 65.84, 62.69, 57.14, 49.05, 41.24, 39.98, 23.50, 22.68, 18.83.

*GlcNAc-anhMurNAc-AF350 **9***. R_f 0.6 (3:2:1 EtOAc:MeOH:H2O); HRMS (ESI-TOF) m/z 816.2601 (816.2604 calcd for $C33H46N5O17S^+$, $[M+H]^+$); ^{1}H-NMR (600 MHz, MeOD) δ 8.03 (s, 1H), 6.55 (s, 1H), 5.25 (s, 1H), 4.41 – 4.39 (m, 2H), 4.01 (q, J = 6.7, 1H), 3.94 (d, J = 7.5, 1H), 3.87 – 3.81 (m, 2H), 3.66 (ddd, J = 17.4, 15.6, 7.1, 3H), 3.57 – 3.51 (m, 4H), 3.44 (dd, J = 11.7, 6.8, 2H), 3.38 – 3.31 (m, 2H), 3.29 (d, J = 0.6, 4H), 2.37 (s, 3H), 1.98 (s, 4H), 1.96 (s, 3H), 1.30 (d, J = 6.7, 4H); ^{13}C-NMR (151 MHz, MeOD) δ 176.04, 174.97, 173.40, 173.30, 164.22, 156.43, 152.58, 150.34, 126.85, 126.70, 115.68, 110.83, 102.20, 102.07, 101.94, 79.21, 78.12, 77.26, 75.85, 75.35, 74.78, 72.06, 65.83, 62.68, 57.21, 49.05, 40.12, 39.87, 35.41, 23.49, 22.67, 18.65, 15.54.

Bacterial Strains and Culture Conditions. For routine cloning and plasmid propagation, DH5α (F^- Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ^-) or TOP10 (F- *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ *lac*X74 *rec*A1 *ara*D139 Δ(*araleu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G*)* were used as hosts. *E. coli* BW25113 (CGSC No. 7636) and MG1655 (CGSC No. 6300) were utilized for gene modification and was obtained from the Coli Genetic Stock Centre (Yale University, New Haven, CT, USA). *Pseudomonas aeruginosa* PAO1 was used for chromosomal DNA isolation and was generously provided by Dr. A. Oliver (Instituto Universitario de Investigación en Ciencias de la Salud (IUNICS), Palma de Mallorca, Spain). If not otherwise stated, cells were grown aerobically in LB medium (10

g/L tryptone, 5 g/L yeast extract, 5 g/L sodium chloride) with the following additions: 150 µg/mL ampicillin, 35 µg/mL kanamycin and/or 35 µg/mL chloramphenicol and 0.2% (w/v) arabinose. For β-lactamase assays, the cells were grown in M9CA supplemented with 1 µg/mL thiamine, 50 µg/mL uracil, 35 µg/mL chloramphenicol, 70 µg/mL kanamycin, 0.5% (v/v) glycerol and arabinose at the indicated concentration.

Deletion of Ec-AmpG from the Chromosome of BW25113 and MG1655. Wild-type *E. coli* BW25113 (CGSC No. 7636) and its isogenic Δ*ampG790::kan* derivative (CGSC No. 8587, Keio Collection No. JW0423-1, Baba *et al.,* 2006) were obtained from the Coli Genetic Stock Center. The kanamycin resistance gene of the mutant was eliminated via recombination between the flanking FRT sites using FLP recombinase (Datsenko *et al*., 2000) supplied by the chloramphenicol and ampicillin resistant, temperature-sensitive plasmid, pCP20 (Cherepanov *et al.*, 1995). This plasmid was subsequently cured by growth at 42 °C and the strain was tested for loss of all antibiotic resistance determinants. Chromosomal deletion of the *ampG* gene in *E. coli* MG1655 (CGSC No. 6300) was carried out using the strategy as described (Baba *et al.*, 2006). Briefly, PCR products were amplified using primers ECΔAMPGF: 5'-GATTACTACAGCTAAATAATATTTACAGATTACGTCAGATGCGTTTTTCGattccggggatccgtcgacc-3' and ECΔAMPGR: 5'-TTAATTTCTGCCCTCTGGCCCGGTGCAAGCCGG-GCCTGTAGACGCCCATGtgtaggctggagctgcttcg-3' and Phusion High Fidelity DNA polymerase as specified by the manufacturer. These primers were designed to have 20 nucleotide C-terminal ends for priming the FRT sites flanking the kanamycin resistance cassette in pKD13[2] (lower case letters) and 50 nucleotide ends homologous to the regions upstream and downstream of the targeted gene deletion (upper case letters).Chromosomal integrants were selected as kanamycin resistant colonies after electroporation of the PCR product into MG1655 harbouring the ampicillin resistant λRed expression plasmid, pKD46 (Datsenko *et al*., 2000). The kanamycin resistance gene was then eliminated using pCP20 (Cherepanov *et al.*, 1995) as described above. The presence of the null deletion in both strains was verified by PCR amplification using the primers ECAMPGF: 5'-GACAACCATCCCAAACTTTTATTGTATTTCCTG-3' and ECAMPGR 5'- CGTCACCGTAAGCATTAATGGTGC-3'.

Construction of pBAD-Pa-AmpG-Km and pBAD-Pa-AmpP-Km. Phusion High Fidelity DNA polymerase was used to PCR amplify the *ampG* (*Pa*4393) and *ampP* (*Pa*4218) ORFs utilising chromosomal DNA isolated from *Pseudomonas aeruginosa* PAO1 as described (Chen *et al.*, 1995). Primers used for amplification were PA4393F: 5'-GATATAgaattcACCATGACTCAGCAATCCTGGCGAGAG-3' (an introduced EcoR1 site is in lower-case letters) and PA4393R: 5'-CTATATaagcttTCAGTGCTGCTCG-GCGTTCTG-3' (an introduced HindIII site is in lower case letters), and PA4218F: 5'-GATATAgaattcACCATGCTTGAGCTGTAC-3' (an introduced EcoR1 site is in lower-case letters) and PA4218R: 5'-CTATATaagcttTCAGGCCTCTTCCGCCCG-3' (an introduced HindIII site is in lower-case letters), respectively. The purified PCR products were then directionally cloned into pBAD322-Km (Cronan, 2006) under the arabinose-inducible, glucose-attenuable P_{BAD} promoter, as EcoR1/HindIII fragments and the fidelity of the inserts was confirmed by DNA sequencing at the Toronto Hospital for SickKids, Centre for Applied Genomics, DNA Sequencing/Synthesis Facility.

Construction of pBAD-Pa-AmpG-V5His-Km and pBAD-Pa-AmpP-V5His-Km. Initially, a fragment containing the C-terminal V5 epitope and six histidine tag, together with the downstream *rrn*B T_1 and T_2 transcriptional terminators was amplified by PCR utilizing pBAD-TOPO/lacZ/V5-His plasmid DNA as a template. Primers V5HISF: 5'-GATATAaagcttGGTAAGCCTATCCCTAAC-3' (an introduced HindIII site is in lower-case letters) and V5HISR: 5'-CTATATtcatgaGAGTTTGTAGAAACGCAAAAAG-3' (an introduced BspH1 site is in lower-case letters) were used, as well as Phusion High Fidelity DNA polymerase under the reaction conditions as suggested by the manufacturer. The resulting purified PCR product was then cloned into pBAD322-Km (Cronan, 2006) expression vector as a HindIII/BspH1 fragment yielding the pBAD322-V5His-Km construct. pBAD-Pa-AmpG-V5His-Km and pBAD-Pa-AmpP-V5His-Km were generated essentially as described above, but the reverse primers used for PCR amplification (AMPGV5HISR: 5'-CTATATaagcttGTGCTGCTCGGCGTTCTG-3' and AMPPV5HISR: 5'-CTATATaagcttGGCCTCTTCCGCCCG-3') were designed to eliminate the native stop codon of both ORFs to preserve reading through the C-terminal V5 epitope and six histidine tags provided by the pBAD322-V5His-Km vector. DNA sequencing at the

Toronto Hospital for SickKids, Centre for Applied Genomics, DNA Sequencing/Synthesis Facility, confirmed the fidelity of all inserts.

Western Blot Analysis. 50 µg of total protein in a final volume of 20 µL was mixed with 20 µL of standard 2X loading buffer and heated at 37 °C for 15 min. Proteins were resolved on 12% polyacrylamide gels and transferred onto Immobilin-P PVDF Western blotting membrane using the Bio-Rad Mini-Trans-Blot Electrophoretic Wet Transfer Cell according to the manufacturer's instructions. The membranes were blocked overnight at 4 °C in 5% skim milk powder dissolved in 1X TBS (2.4 g/L Tris, 8.8 g/L NaCl, pH 7.4), then washed twice in 1X TBS for 5 min, prior to incubation in the presence of mouse α-V5-HRP conjugate at a dilution of 1:5000 in 1% skim milk powder dissolved in 1X TBS for 1 hr at room temperature. The blots were then rinsed four times in 1X TBS containing 0.05% Tween-20 for 10 min at room temperature and immunoreactive proteins were detected using Luminata Forte Western HRP Chemiluminescent substrate, as recommended by the supplier. Fluorchem 8900 was used to obtain the resulting signal.

Dose-dependent expression of V5His tagged *Pa*-AmpG and *Pa*-AmpP. Overnight cultures of *Ec*MGΔ*ampG* transformed with the pBAD-*Pa*-AmpG-V5His-Km or pBAD-*Pa*-AmpP-V5His-Km were inoculated, one in fifty, into 3.0 mL of the LB containing 35 µg/mL kanamycin, and grown at 28 °C until the optical density at 600 nm reached approximately 0.4. To induce expression, L-arabinose was added at the indicated concentration and the cultures were grown at 28 °C for an additional three hours. The cells were then pelleted by centrifugation at 6,000 rcf at 4 °C for 5 min, washed once with PBS, resuspended in lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton-X-100, 100 µg/mL lysozyme, 50 µg/mL DNase, 0.1 mM PMSF and 0.5 mM TCEP, then mixed by end-over-end rotation for 1 hr at 4 °C. The cellular debris was subsequently removed by centrifugation at 12,000 rcf at 4 °C for 30 min, and the supernatants, which contained solubilized membranes, were analysed by Western blot analysis as described above. Protein concentrations were quantified by the Bradford assay using bovine gamma globulin as the standard.

Spheroplast fractionation. Approximately 60 mg of spheroplasts were collected by centrifugation at 2,000 rcf at 4 °C for 5 min, washed once in dilution solution containing

0.5 mM TCEP and 0.1 mM PMSF, resuspended in the same buffer, supplemented with 1% Triton-X-100 and 0.1% SDS, then mixed by end-over-end rotation for 1 hr at room temperature. The suspensions were then pelleted by two consecutive spins at 16,000 rcf at 4 °C for 20 min and the resulting supernatants containing solubilized membrane protein were retained. The final pellets, representing the insoluble fraction, were resuspended in 300 µL of dilution solution, sonicated three times at 50% duty cycle, six second bursts, with 10 seconds of incubation on ice between each burst. Western blot analysis was performed as described above. The Protein concentration of all fractions was quantified by the Bradford assay using bovine gamma globulin as the standard.

Transport of Probe 9 by Native *Ec*BW and *Ec*BWΔ*ampG E. coli.* Wild-type or isogenic *ampG*$^-$ BW25113 *E. coli* (*Ec*BW or *Ec*BWΔ*ampG*) were grown until the OD600 reached ~ 0.5 - 0.6, as described in the 'Preparation of Spheroplasts' portion of the Materials and Methods. To 1 L of LB, preheated to 37 °C, was added 120 mL of OD600 0.5 - 0.6 culture. The cells were grown aerobically at 37 °C for 1 hr, centrifuged at 3000 rcf at 4 °C for 10 min, and resuspended to homogeneity in dilution solution to a concentration of 0.25 g/mL. The resuspended bacteria was stored at 4 °C. The next day 200 µL of 0.25 g/mL *Ec*BW or *Ec*BWΔ*ampG* was added to microcentrifuge tubes, and the solution was brought up to 250 µL with dilution solution. The experiment was initiated by the addition of 100 µL of 175 µM probe **9**. Blanks were prepared in which dilution solution was added in place of probe **9**. Tubes were mixed by inversion several times, then incubated for 1 or 3 hr at 25 °C in the dark using a digital heatblock. Final reaction conditions were: 350 µL; 50 µM probe **9**, and 0.15 g/mL *E. coli*. Post-experiment washing and fluorescence quantitation was performed as described in the Materials and Methods.

Time Course of Probe 9 Uptake by *Ec*BW spheroplasts. The experiment was carried out in duplicate using *Ec*BW spheroplasts. In brief, 50 µL of 0.25 g/mL spheroplasts were added to microcentrifuge tubes, followed by the addition of 50 µL of 100 µM probe 9 in dilution solution, beginning with those tubes corresponding to the longest time points. Blanks contained dilution solution in place of probe 9. The tubes were inverted several times to mix, and were incubated for 0, 5, 10, 30, 60, 90, 120, and 240 minutes at 25 °C in the dark using a digital heatblock. Final reaction conditions were: 100

230 µL; 50 µM probe 9, and 0.125 g/mL spheroplasts. Post-assay spheroplast washing and fluorescence quantitation was performed as described in the Materials and Methods.

Microscopy. Images of post-assay spheroplasts were captured using an Axioskop 2 Plus light microscope equipped with an Axiocam camera, and were processed using Axiovision software. Fluorescence images were obtained using a Colibri LED light source. 235 A 2 sec exposure was used in all fluorescence images for comparability. For the NBD-Glc images, brightness was adjusted post-capture, as fluorescence intensity rapidly decayed on excitation. Identical brightness settings were used for all NBD-Glc fluorescence images for comparability. Excitation wavelengths of 365 nm and 470 nm were used for probe **9** and NBD-Glc, respectively. Both DIC and fluorescence images 240 were captured at 1000 times magnification.

Supporting References

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