Supporting Information

Surface Display of a Redox Enzyme and its Site-Specific Wiring to Gold

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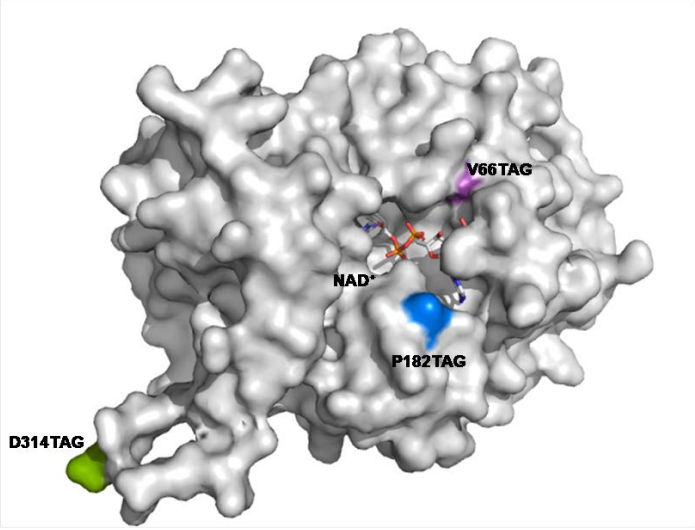

Figure S1: Three-dimensional model of ADHII from *Z. mobilis*, with its NAD^+ cofactor. Mutation sites: V66, P182 and D314 are highlighted.

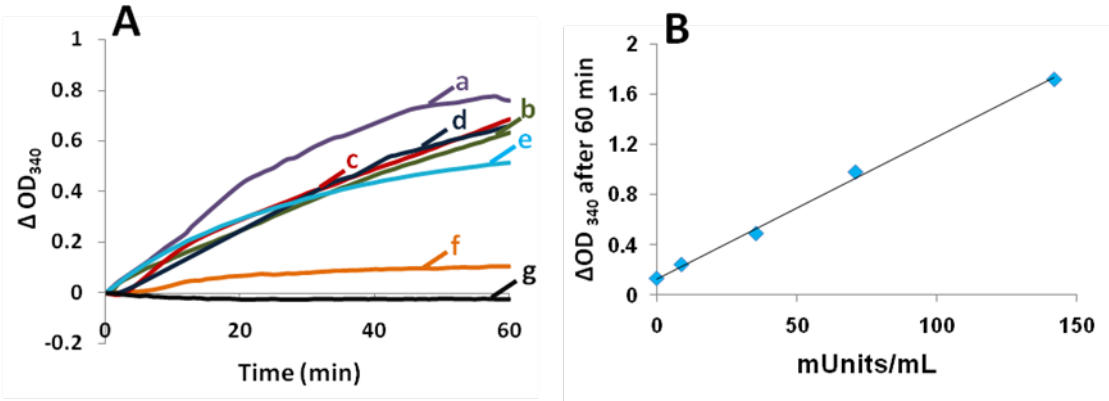

Figure S2: (**A**)-ADHII activity of the Az-Phe incorporated mutants, based on NADH absorbance upon addition of ethanol. (a) *E. coli* displaying ADHII; (b) *E. coli* displaying ADHII V66TAG; (c) *E. coli* displaying ADHII P182TAG; (d) *E. coli* displaying ADHII D314TAG; (e) purified ADHII (35 mUnits/ml); (f) WT *E. coli*; (g) *E. coli* displaying ADHII, without ethanol. (**B**)-Calibration curve of ADHII activity, based on purified ADH.

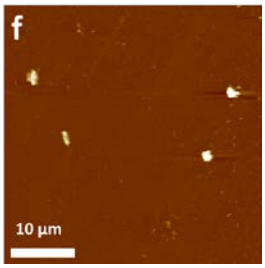

Figure S3: Atomic Force Microscopy image (40x40 µm) of *E. coli* P182TAG ADHII upon electrochemical detachment.

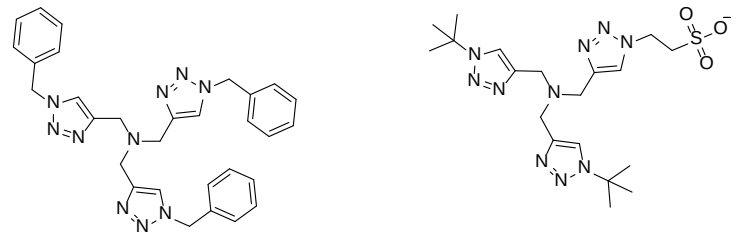

Figure S4: Chemical structures of the non-biocompatible (**4**) and biocompatible (**5**) copper ligands for the 'click' alkyne-azide cycloaddition.

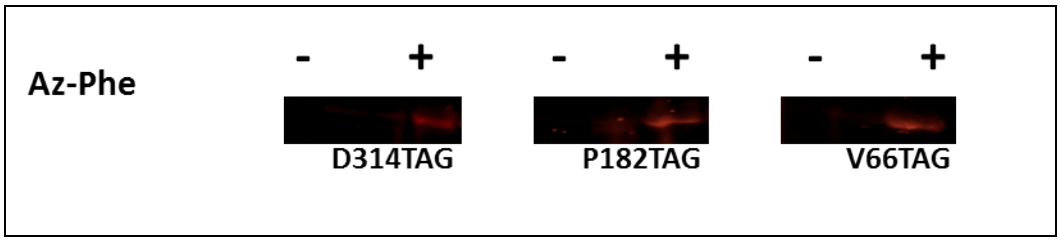

Figure S5: Fluorescent SDS-PAGE of *E. coli* outer membrane fractions from three mutants grown in the presence (+) or absence (-) of Az-Phe, upon a 'click' reaction with **3.**

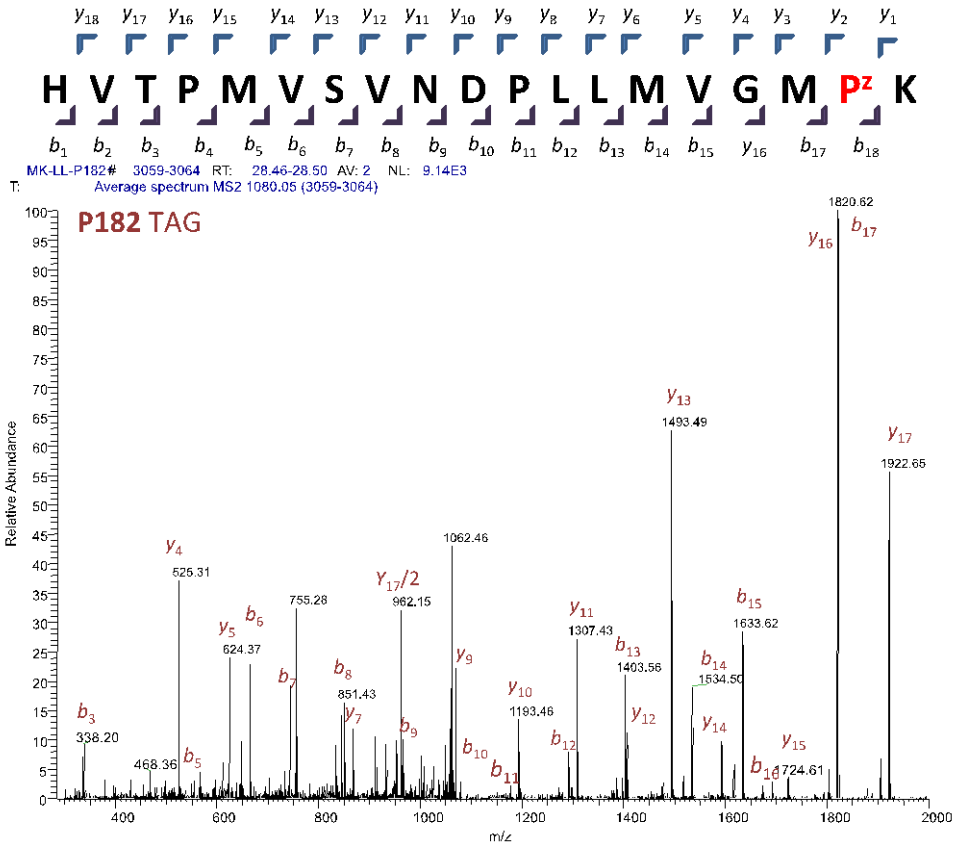

Figure S6: MS/MS spectrum of the peptide from P182TAG, containing Az-Phe (labeled as P^z). The expected fragment ion masses are shown in the spectrum.

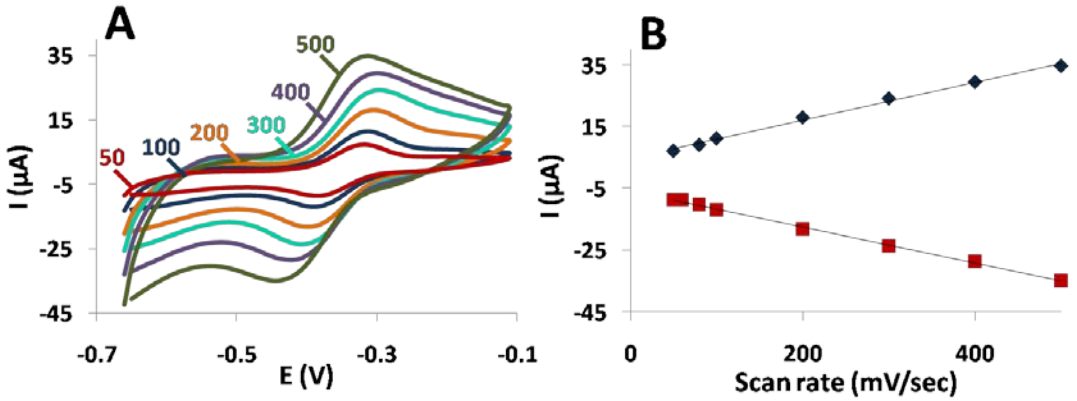

Figure S7: (A)-Cyclic voltammograms of a gold disc electrode, modified with **2** under different potential scan rates (in mV/sec) and **(B)-** Peak current dependence on the potential scan rate, red - cathodic peak current, blue - anodic peak current. Middle point potential is -350 mV vs. Ag/AgCl electrode.

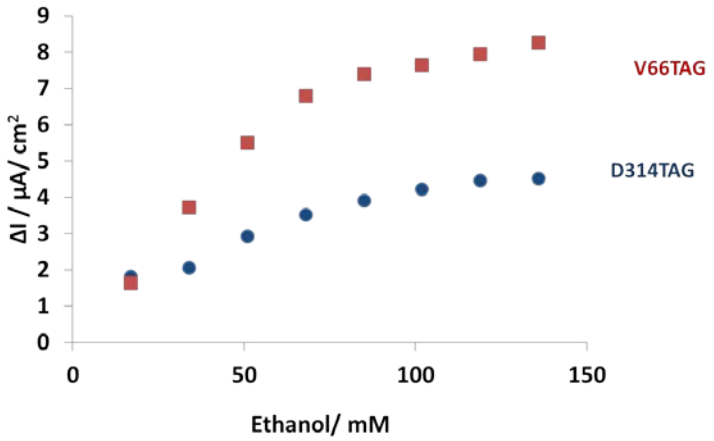

Figure S8: Amperometric response of *E. coli* attached to a gold working electrode, upon a 'click' reaction with **2**, under applied potential of -0.3V (vs. Ag/AgCl) to changing ethanol concentrations. (ca. 150mM equals 1% ethanol in our system).

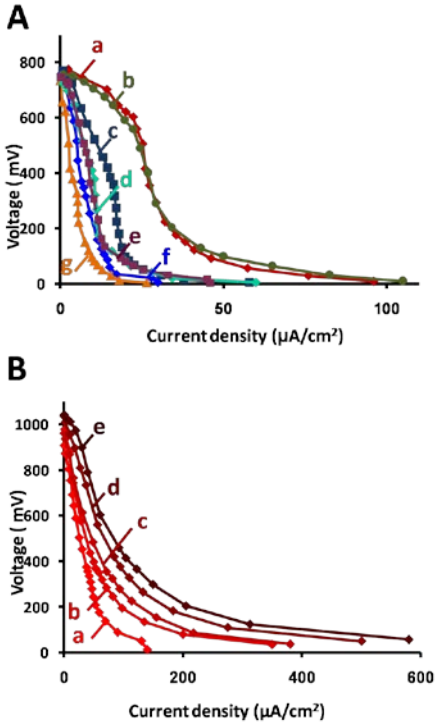

Figure S9: (A) Polarization curves of fuel cells constructed with *E. coli* attached to gold coated plates, following 'click' reaction with **2**. (a) *E. coli* V66TAG. (b) *E. coli* P182TAG. (c) *E. coli* D314TAG. (d) *E. coli* harboring pJM7-ADHII, non-specifically attached to an electrode. (e) Purified ADHII, non-specifically attached to electrode. (f) *E. coli* harboring pJM7-ADHII, in solution. (g) WT *E. coli* in solution. (B) Polarization curves of a fuel cell constructed with wired *E. coli* V66TAG over a week of operation. After (a) one day. (b) two days. (c) 4 days. (d) 6 days. (e) 7 days. Measurements were performed under ambient temperature, in the presence of 2% ethanol. A potential of +700 mV vs. Ag/AgCl was applied on the cathode.

Methodology*:*

Strains, plasmids and reagents

E. coli JK321, which carries a mutant OmpT gene silencing proteolytic activity of the OmpT outer membrane protease and the autodisplay plasmid pJM7 were kindly provided by Prof. Thomas Meyer, Max Planck Institute, Germany. Genomic DNA from *Zymomonas mobilis* was generously supplied by Prof. Yuval Shoham, the Technion, Israel Institute of Technology. The plasmid pSup-MjAzRS-6TRN was generously donated by Peter G. Schultz, The Scripps Research Institute, La Jolla, CA.

All chemicals and reagents were purchased from Sigma-Aldrich (Rehovot, Israel) or Acros (Geel, Belgium) and used without further purification. The unnatural amino acid para-azido-L-phenylalanine was purchased from Chem Impex, Wood Dale, IL.

PCR was performed with Kapa HiFi PCR Kit (Kapa Biosystems, Woburn, MA). Site directed mutagenesis was performed using the QuickChange II kit (Santa Clara, CA). Plasmid DNA isolation was performed with the HiYield Plasmid Mini Kit (RBC Bioscience, Chung Ho City, Taiwan). Oligonucleotides were supplied by IDT, Jerusalem, Israel.

Cloning

The alcohol dehydrogenase II gene (1146bp) was amplified from *Zymomonas mobilis* genome by polymerase chain reaction (PCR) using purified genomic DNA as a template, with the following primers:

Forward (pjm-ZADH2-F (containing an underlined *Xho*I restriction site)):

CTCGCACGTGCTCGAGGCTTCTTCAACTTTTTATATTC

Reverse (pjm-ZADH2-R (containing an underlined *Kpn*I restriction site)):

ACATAAGCGGTACCGAAAGCGCTCAGGAAGAGTTC

PCR products and the autodisplay vector pJM7 (4652 bp), were digested with *Xho*I and *Kpn*I (all restriction enzymes supplied by Fermentas, Leon-Rot, Germany), and the ADHII gene was ligated into the autodisplay vector, pJM7 which resulted in a new plasmid pJM7-ADHII. pJM7-ADHII was transformed into competent *E. coli* strain DH5α by electroporation. The plasmid was purified, sequenced and introduced into the competent *E. coli* strain JK321. All cloning steps were performed by standard methods.

Site directed mutagenesis

In order to identify potential templates for homology modeling of *Z. mobilis* ADHII its sequence was submitted to the HHprep server[1]. Similarity searches revealed that 1,3-propanediol dehydrogenase from *Thermotoga maritima* (PDB ID: 1O2D) were used as a template for modeling (P value = 2.2E-79). Finally, the generation of the ADHII three-

dimensional model was built with the M4T[2] approach using MODELER[3]. Mutation sites V66 and P182 were selected for their close proximity to the NAD^+ binding site (<10Å), and to minimize disruption to residues crucial to the coordination of cofactors (figure **S1**). Additionally, a distant (negative) control mutant D314 was selected, as this residue is too distant from the cofactor binding site to participate in electron transfer (>40 Å away).

Site-directed mutagenesis on the pJM7-ADHII was performed using a QuickChange II kit according to manufacturer's instructions, using the following primers for PCR, containing the underlined mutation site. Table 1 describes the primers that were designed in order to produce the 3 different mutants, V66TAG, P182TAG and D314TAG.

Table 1: Primers for mutagenesis

Mutant	**Direction**	**Primer**	**Sequence**
V66TAG	Forward	QC-ZADH V66F	CTGCTGTTTATGATGGCTAGATGCCGAACCCGACTGTTACCG
V66TAG	Reverse	QC-ZADH V66R	CGGTAACAGTCGGGTTCGGCATCTAGCCATCATAAACAGCAG
P182TAG	Forward	QC-ZADH P182F	GTTGATGGTTGGTATGTAGAAAGGCCTGACCGC
P182TAG	Reverse	QC-ZADH P182R	GCGGTCAGGCCTTTCTACATACCAACCATCAAC
D314TAG	Forward	QC-ZADH D314F	CGATATCGCCAATCTCGGTTAGAAAGAAGGCGCAGAAG
D314TAG	Reverse	QC-ZADH D314R	CTTCTGCGCCTTCTTTCTAACCGAGATTGGCGATATCG

The pJM-ADHII (carrying the mutated ADHII gene) and pSup-MjAzRS-6TRN plasmids were co-transfected into the *E. coli* strain JK321 by standard heat shock procedure.

Surface display of ADHII

E. coli JK321 harboring the plasmid pJM7-ADHII were grown under selective conditions (kanamycin and carbenicillin). The activity of ADHII, displayed on the bacteria surface, was examined both by a colorimetric assay and by an electrochemical assay

Incorporation of p-azido-L-Phenylalanine (Az-Phe)

E. coli JK321 containing both pSup-MjAzRS-6TRN and the site-mutated pJM7-ADHII plasmids were grown in Luria-Bertani (LB) broth at 37 °C under selective conditions (carbenicillin, kanamycin and chloramphenicol) until an OD_{600} of 0.5-0.7 was reached. Az-Phe was then added to a final concentration of 1 mM. Cells were grown at 37 °C in the presence of the unnatural amino acid for 14 h. The cells were centrifuged at 4000 rpm for 15 minutes in 4 °C and washed three times with PBS.

Biochemical measurements

The alcohol dehydrogenase activity of intact cells was determined by monitoring the conversion of NAD^+ to NADH (figure **S2**), based on the enzymatic alcohol dehydrogenase assay. Cells were washed twice with PBS then resuspended in the same buffer, to which NAD^+ was added to a final concentration of 5 mM. Measurements were performed using UV-star 96-well plate (Grenier Bio-one, Germany). To each sample (200 µL final volume)

ethanol was added to a final concentration of 20%. Absorbance was measured at 340 nm for one hour, in a microplate reader (BioTek instruments, Winoosky, VT).

Bacterial surface digestion

Surface proteins were digested following a published procedure[4], with slight modifications. A 200 mL culture of *E. coli* expressing Az-Phe surface displaying ADHII were harvested and washed 3 times with PBS.

For trypsin digestion experiments the cells were resuspended in 1 mL PBS containing 40% sucrose and 5 mM dithiothreitol, to which was added 10 mg of trypsin gold (Promega, Madison, WI). The mixture was incubated at 37°C for one hour, then the cells were pelleted and the supernatant filtered using 0.22 μm pore size filters. Formic acid was added to a final concentration of 0.1%, in order to stop proteolysis, and the samples were purified using a Zip-tip® (Millipore, Milford, MA).

Peptide MS measurements

The peptides were separated on-line by nano-flow reverse-phase liquid chromatography. The sample was eluted into an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) at a flow rate of 0.30 μL/min. The mass spectrometer was operated in the data-dependent mode to automatically switch between Orbitrap-MS and MS/MS (MS2) acquisition. Survey full scan MS spectra (from *m/z* 300 to 2000) were acquired in the Orbitrap with resolution R = 60,000 at *m/z* 400.

Linkers 2 and 3 synthesis

Full electrochemical characterization of **2** on a gold disk electrode has been performed according to Katz at al[5], shown in figure **S6.**

Thin-layer chromatography was performed on TLC aluminum sheets silica gel 60 with F254 indicator (Merck). NMR analysis was performed using a Bruker Advance DMX500 spectrometer. Compounds were identified by UV detection with dual wavelengths (230 nm and 260 nm). Mass spectrometry analyses were performed on a LCQ Fleet mass spectrometer (Thermo-Finnigan, San Jose, CA) with an ESI source. Spectra were collected in the positive ion mode and analyzed by Xcalibur software (Thermo-Finnigan, San Jose, CA).

Synthesis of 2-chloro-3-propargylaminonaphtoquinone

2,3-dichloro-1,4-naphtoquinone (1.8 g, 8 mmol) and propargyl amine (860 mg, 16 mmol) were added to 100 mL ethanol in a round bottom flask. The mixture was heated to 50 °C until complete dissolution. The reaction was stirred for one hour at room temperature. The solid

residue was filtered, washed with cold hexane and dried, yielding 2-chloro-3-propargylaminonaphtoquinone as orange crystals (1.29 g, 5 mmol, 63%).

^{1}H-NMR (500 MHz, $CDCl_3$), δ (ppm) 8.16 (d, 1H, *J*=7.72), 8.06 (d, 1H, *J*=7.49), 7.75 (t, 1H, *J*=7.49), 7.66 (t, 1H, *J*=7.72), 4.64 (d, 2H, *J*=2.49), 2.39 (t, 1H, *J*=2.49).

MS (ESI): *m/z* $[M+H]^+$ 246.04

Synthesis of 2-mercapto-3-propargylaminonaphtoquinone (2)

Sodium sulfide (6.3 gr, 82 mmole) was added to a 20% water in THF solution containing 2-amino-3-chloro-1,4-naphthoquinone (200 mg, 0.8 mmole). The reaction mixture was warmed to 80 °C for 3 h, then cooled and acidified with HCl. The solvent was reduced under vacuum, and the mixture was extracted with chloroform and dried over magnesium sulfate. Filtration, followed by solvent removal under vacuum, yielded **2** as a dark purple powder (138 mg, 0.5 mmol, 63%).

^{1}H-NMR (500 MHz, $CDCl_3$), δ (ppm): 8.05 (d, 1H, *J*=7.72), 8.01 (d, 1H, *J*=7.49), 7.67 (t, 1H, *J*=7.79), 7.6 (t, 1H, *J*=7.72), 5.57 (s, 1H), 5.37 (s, 1H), 4.12 (d, 2H, *J*=2.49), 2.27 (t, 1H, *J*=2.49). MS (ESI): *m/z* $[M+H]^+$ 244.06

Synthesis of 5-(4)-carboxytetramethylrhodamine-N-propargylamine (3)

TAMRA (5-(and-4)-carboxytetramethylrhodamine) was synthesized following a combination of procedures described by Kvach et al[6] and Clark et al.[7]

5-(and-4)-carboxytetramethylrhodamine (86 mg, 2 mmol), propargyl amine (7.15 mg, 0.13 mmol), HBTU (75.8 mg, 0.2 mmol), and TEA (56.1 μL) were dissolved in 1mL DMF. The reaction mixture was stirred at room temperature overnight, the solvent was removed under reduced pressure, and the compound wad purified by RP-HPLC (from 90:10 to 10:90 in 30 min, 0.1% TFA) to afford a mixture of isomers 5 and 4 (3:1) as dark-red solid (11 mg, 18%).

^{1}H NMR (400 MHz, METHANOL-d_4), δ = 8.88 (br, s, 1H), 8.76 (d, *J*=1.7 Hz, 1 H), 8.39 - 8.45 (m, 1 H), 8.24 (dd, *J*=7.9, 1.8 Hz, 1 H), 8.17 (dd, *J*=5.5, 3.8 Hz, 1 H), 7.96 (dd, *J*=7.7, 0.7 Hz, 1 H), 7.56 (br, s, 1 H), 7.51 (d, J=7.93 Hz, 1 H), 7.12 (d, , *J*=9.6 Hz, 2 H), 7.03 (dd, *J*=9.6, 2.4 Hz, 2 H), 6.96 (d, *J*=2.44 Hz, 3 H), 4.42 (d, *J*=2.5 Hz, 2 H), 4.22 (d, *J*=2.5 Hz, 2 H), 2.65 (t, *J*=2.5 Hz, 1 H), 2.63 (t, *J*=2.5 Hz, 1 H) ppm.MS (ESI) m/z 468.1 $[M]^+$.

'Click' Reaction conditions:

Cu(I) catalyzed alkyne-azide cycloaddition

Reactions were performed according to the procedure published by Cravatt and co workers[8]. Cultures (10 mL) were grown in the presence of Az-Phe as described, collected and washed three times with PBS, then resuspended in 500 μL of the same buffer. Compound **2** was

added to a final concentration of 50 μM, tris(2-carboxyethyl)phosphine (TCEP), tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA), and $CuSO_4$ were added to a final concentrations of 1 mM, 100 μM and 1 mM, respectively. The reaction mixture was incubated in room temperature for 6 h, and the cells pelleted and washed three timed with PBS.

Biocompatible Cu(I) catalyzed alkyne azide cycloaddition

Bis(tert-butyltriazolyl) ligand (BTTES) was synthesized according to a published procedure by Soriano del Amo et al.[9]. Cultures (10 mL) were grown in the presence of Az-Phe. The cells were pelleted, washed three times with PBS and resuspended in 500 μL PBS. The following materials were added: alkyne **2** to a final concentration of 50 μM, TCEP (2.5 mM), BTTES (300 μM) and $CuSO_4$ (50 μM). The reaction mixture was vortexed and incubated at room temperature with gentle agitation for 15 minutes, and then immediately pelleted and washed three times with PBS.

Live cell assay

Biocompatible and non biocompatible 'click' reactions with **2** were performed on Az-Phe incorporated bacteria. Following centrifugation and washing with PBS, the pellet from 10 mL of culture was resuspended in 1 mL fresh LB (without antibiotics) and incubated at 37 °C for 1 h. After recovery, a 10 μL sample of each culture was added to 990 μL fresh LB, the resulting culture then was used to immediately prepare serial dilutions down to 10^{-8}. From each of these serial dilutions a 50 μL sample was plated on LB agar with carbenicillin, chloramphenicol and kanamycin. Plates were incubated at 37 $^{\circ}$C for 14 h, and the individual colonies were counted.

AFM characterization and preparation

Atomic force microscopy imaging was performed at ambient conditions using a Digital Instrument Dimension 3100 with a standard Dimension SPM head mounted on an active anti-vibration table. Phosphorous-doped silicon RTESP type probes (Veeco instruments, Plainview, NY) were used. Images were acquired at 256 x 256 or 512 x 512 pixels in tapping mode with a scan size of either 5 x 5 μm or 40 x 40 μm and a scan rate of ~1Hz. Bacteria were reacted with **2** as described. The cells were resuspended in 200 μL PBS and incubated on glass slides with a 20 nm gold coating for one hour in room temperature. The slides were washed thoroughly with Millipore water, dried under nitrogen and imaged in tapping mode in the AFM.

Surface regeneration

Electrochemical removal of bacteria attached to a gold coated slide was performed using a VSP potentiostat (BioLogic science instruments, Claix, France). Bacteria were reacted with linker **2**, and incubated for 1 hour on glass slides (with a 5 nm chromium adhesion layer beneath a 20 nm gold layer). The slides were washed thoroughly with doubly deionized water, dried under nitrogen and imaged by AFM to confirm bacterial adhesion. Detachment of bacteria from the surface was achieved by applying -1.0 V (using Ag/AgCl as a reference electrode) for 10 min. The slides were washed, dried and imaged by AFM.

TEM characterization

Transmission electron microscopy studies were performed on a FEI Tecnai 12 G2 Twin TEM (Hillsboro, OR), operated at 120kV. Gold nanoparticles (8 nm ±1) were synthesized according to the published procedure[10]. Mutants were 'click' reacted with **2** as described in the SI. The cells were incubated with 500 µL of the gold nanoparticle suspension for two hours at room temperature. The bacteria were then washed with PBS and diluted 10 times. 1 µL of this suspension was deposited on a carbon coated lacy grid, dried and imaged by TEM, using a Gatan CCD camera for data capture.

Fluorescent SDS-PAGE

Cultures (200 mL) were grown in the presence or absence of **1** as described. The cells were pelleted and washed three times with PBS, and resuspended in 1 mL PBS. 'Click' reaction was performed with alkyne **3** (100 µM), in the presence of BTTES, TCEP and $CuSO_4$ (final concentrations 300 µM, 2.5mM and 50 µM, respectively). The reaction mixture was incubated at room temperature for 30 min, pelleted and washed with PBS. The pellet was resuspended in 2 mL TES buffer (50 mM), lysed by sonication and the lysate centrifuged for 5 min at 13,000 rpm. The supernatant was then centrifuged at 100,000 rpm for 1 h. The pellet was resuspended in 4% SDS in 10 mM Tris, the samples were diluted with 4X SDS sample buffer and heated for 10 min at 70 °C, after which it was loaded and run on a 12% SDS-PAGE gel. Labeled proteins were visualized in-gel using ImageQuant LAS 4000 imager (Fujifilm, Tokyo, Japan)

Electrochemical measurements (electrobiocatalysis)

Biocatalysis assays were performed using a PalmSense potentiostat (Palm Instruments, Houten, The Netherlands) with screen-printed Au (2 mm^2) Pt- pseudo-Ag/AgCl electrodes on ceramics (Bio Sensor Technology, Berlin, Germany). A 2 µL suspension of 'click' reacted cells was deposited onto the gold working electrode. Electrochemical measurements were

performed in 1 mL 0.1 M Tris (pH 7.4), 10 mM $CaCl_2$ in the presence of NAD^+. Scan rates were 5 mV/sec. for the biocatalysis measurements, ethanol was added to a final concentration of 1%.

Fuel cell construction and performance characterization

Polished graphite plates were sputter-coated with gold (100 nm) using Emitech K575X Sputter Coater (Emitech, Ashford, Kent, UK). Cultures (10 mL) were reacted with **2** as described, and incubated with the gold coated graphite electrodes for 1 hour (deposition area-1 cm^2). The electrodes were washed and then served as the anode in a single compartment electrochemical cell (10 mL); consisting 0.1 M Tris buffer (pH 7.4), $CaCl_2$ (10 mM), NAD^+ (5 mM) and ethanol (2%). The cathode was potentiostatically controlled, using three-electrode configuration: gold disk (3 mm diameter) as working electrode, platinum wire as counter electrode and Ag/AgCl as a reference electrode (ALS, Tokyo, Japan). The cathode was biased at a potential of +700 mV against Ag/AgCl[11]. For control experiments, ADH from *Saccharomyces cerevisiae* (Sigma, Israel) solution in PB (50mU/mL) or ADHII expressing bacteria were attached to the gold nonspecifically. Gold electrodes were immersed in a fresh solution of cysteamine (20 mM) in PBS for 1 hour. The electrodes were washed, incubated with bacteria or ADHII in the presence of **2** (50 μM) for 1 hour. Cross-linking was performed using glutaraldehyde (0.1% in PBS) for 3 min. The electrodes were washed and used as anodes in the fuel cell. The voltage generated from the biofuel cells was measured by a manual multimeter (DM-97, HTC-instruments). Various external resistances were applied between the anode and cathode by a resistance decade box (RBOX 408, Lutron Electronic Enterprise, Taipei, Taiwan). The generated voltage at each resistance was measured after reaching equilibrium. Measurements were carried out at ambient temperature.

For the fuel cell long term measurements, the cell compartment contained, additionally, LB and antibiotics (Kanamycin, Carbenicillin and Chloramphenicol) in order to avoid contaminations. The power density curve was measured every 1-2 days; ethanol was added to a final concentration of 2% before each measurement. The power density plot has been recorded as described. Between measurements, the cells were left in room temperature and an external resistance of 20 kΩ was used as an external load.

References

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