Electronic Supplementary Information for:

**Nitrogenase bioelectrocatalysis: heterogeneous ammonia and hydrogen production by MoFe protein**

by

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Figure S1 – Cyclic voltammetry of a BSA control bioelectrode in the absence (dashed line) and presence (solid line) of 50 mM NO$_2^-$, evaluated at a scan rate of 2 mv s$^{-1}$ in HEPES buffer (pH 7.4, 250 mM) in the absence (red lines) or presence (black lines) of 200 µM Cc$^+$. 

Supplementary Figures and Tables
Figure S2 - Cyclic voltammetry of additional control bioelectrodes in the (a) absence or presence of 50 mM (b) N$_3^-$ or (c) NO$_2^-$, evaluated at a scan rate of 2 mv s$^{-1}$ in HEPES buffer (pH 7.4, 250 mM). Complete MoFe protein bioelectrodes (prepared with WT MoFe protein) are compared against bioelectrodes without Cc$^+$/Cc (black dashed line), without polymer and MoFe protein (black dotted line), as blank glassy carbon (GC) electrodes only (red solid line) or without Cc$^+$/Cc and MoFe protein (red dashed line).
Figure S3 – Bulk bioelectrosynthetic reduction of 50 mM (a) N\textsubscript{3} and (b) NO\textsubscript{2} by β-98\textsuperscript{Yrr→His} MoFe protein bioelectrodes (black lines), operating in stirred HEPES buffer (pH 7.4, 250 mM) containing 200 μM Cc\textsuperscript{+} (\textit{E}_\text{applied} = -1.25 \text{ V vs. SCE}). Control bioelectrodes (red lines) were prepared using apo-MoFe protein (red lines). Substrates were injected at 120 s.
Figure S4 – Calibration standards for NH$_3$ using ortho-phthalaldehyde (detailed below, $n = 3$).
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Corrected Current Densities (µA cm⁻²)</th>
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<tbody>
<tr>
<td></td>
<td>Wild-type MoFe</td>
</tr>
<tr>
<td>H⁺</td>
<td>61 ± 16</td>
</tr>
<tr>
<td>N₃⁻</td>
<td>128 ± 21</td>
</tr>
<tr>
<td>NO₂⁻</td>
<td>210 ± 57</td>
</tr>
</tbody>
</table>

**Table S1** – Catalytic current densities extracted from cyclic voltammograms of respective MoFe protein bioelectrodes (-1.35 V vs. SCE) operating at 2 mV s⁻¹ in HEPES buffer (pH 7.4, 0.2 M) containing 200 µM Cc⁺ (n = 3, mean ± SD). Catalytic current densities are reported corrected to corresponding current densities obtained from apo-MoFe protein control bioelectrodes (errors propagated).
Materials and Methods

Chemicals
All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless specified otherwise. Poly(vinylamine) and ethylene glycol diglycidyl ether (EGDGE) were purchased from PolySciences, Inc. Saturated calomel (SCE) reference and glassy carbon working electrodes were purchased from CH Instruments, Inc.

Electrochemical analysis
All electrochemical analyses were performed anaerobically (<1 ppm O\textsubscript{2}) to avoid competitive O\textsubscript{2} reduction at the working electrode and to preserve nitrogenase MoFe protein activity. HEPES buffer (pH 7.4) was used as the supporting electrolyte at a concentration of 250 mM to avoid migration issues associated with N\textsubscript{3}\textsuperscript{-} and NO\textsubscript{2}\textsuperscript{-}. Electrochemical measurements were performed using a CH Instruments (Inc.) potentiostat (1230b), utilizing a large Pt counter electrode in a traditional 3-electrode configuration.

Cell growth and nitrogenase MoFe protein purification
*Azotobacter vinelandii* strains DJ995 (wild-type MoFe protein) and DJ1003 (apo-MoFe protein) were grown and polyhistidine-tagged proteins were purified as previously reported.\textsuperscript{1} Similarly, strain DJ939 (β-98\textsuperscript{Ty r→His}) was grown and the corresponding non-His tagged MoFe protein was purified as described. Protein manipulation was either performed under an argon atmosphere in septum-sealed vials (using a Schlenk line) or within an anaerobic tent (<1 ppm O\textsubscript{2}, Coy Laboratory Products, MI, USA).

Nitrogenase MoFe proteins were assayed for H\textsuperscript{+}, C\textsubscript{2}H\textsubscript{2} and N\textsubscript{2} activity.

<table>
<thead>
<tr>
<th>MoFe Protein</th>
<th>H\textsubscript{2}</th>
<th>C\textsubscript{2}H\textsubscript{4}</th>
<th>NH\textsubscript{3}</th>
</tr>
</thead>
<tbody>
<tr>
<td>DJ995</td>
<td>2493</td>
<td>2349</td>
<td>606</td>
</tr>
<tr>
<td>DJ939</td>
<td>1100</td>
<td>600</td>
<td>220</td>
</tr>
</tbody>
</table>

MoFe protein bioelectrode preparation
Purified MoFe protein (20 mg mL\textsuperscript{-1}, 15 µL) was thawed anaerobically and immobilized at the surface of a glassy carbon electrode by mixing with poly(vinylamine) (10 mg mL\textsuperscript{-1}, 15 µL) and EGDGE (10% v/v, 2 µL); 5 µL of this mixture was applied to the surface of each glassy carbon electrode and dried under reduced humidity for 1 hour, prior to testing.

The ratio of polymer:crosslinker is of importance; too little crosslinker results in an unstable film, whereas excessive crosslinker also results in an unstable film whereby excessive EGDGE on the electrode surface results in a film that does not completely dry (EGDGE is a liquid at room temperature). Since EGDGE will crosslink between polymer-polymer, protein-protein and protein-polymer, the concentration of EGDGE also depends on the loading of protein per electrode. For the immobilization of nitrogenase MoFe protein within a poly(vinylamine) film, an approximate concentration of 23 mol%
EGDGE to poly(vinylamine) was utilized, resulting in approximately one epoxide functionality to every two primary amines present on the poly(vinylamine) backbone (where a 10 mg mL⁻¹ solution of poly(vinylamine) approaches saturation). Previous work employing redox polymers and using EGDGE as the crosslinker indicate a concentration of between 20-30 mol% EGDGE to be optimal.²³ Finally, a volume of 5 µL of the final enzyme/polymer/crosslinker mixture was applied to the surface of a 3 mm glassy carbon electrode; this volume ensure the complete yet non-excessive coverage of the surface of the working electrode.

Ammonia/ammonium detection assay
NH₃ was quantified as previously reported.⁴⁵ An NH₃ detection reagent was prepared by mixing phosphate buffer (pH 7.3, 0.2 M, 100 mL), 2-mercaptoethanol (25 µL) and ortho-phthalaldehyde (270 mg dissolved in 5 mL of ethanol) and storing in the dark. Following bioelectrosynthetic experiments, 250 µL of the electrolyte solution (containing NH₃ produced by MoFe protein bioelectrodes) was mixed with 1000 µL of the NH₃ detection reagent and incubated for 30 min in the dark. NH₃ was quantified by exciting the solutions at 410 nm and measuring their emission readings at 472 nm. All samples were quantified as triplicates and corrected against background samples prepared with apo-MoFe protein bioelectrodes.

REFERENCES