**Supplementary File 1A: Primers, plasmids and strains used in this study**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Strain** | **Plasmid** | **Target gene** | **Genetic tag** | **Primers used for cloning** | |
| **Name** | **Sequence** |
| M7-2 | pCB16-1 | E. coli AdhE | N-histag | 1184 | tgagatccggctgctaacaaa |
| 1252 | ccggatatagttcctcctttcag |
| 1524 | CATGGTATATCTCCTTCTTAAAGTTAAACAA |
| 1710 | taattttgtttaactttaagaaggagatataccatgcaccaccaccaccaccacgagaacctgtatttccagggtatgGCTGTTACTAATGTCGCTGA |
| 1711 | ctcagcttcctttcgggctttgttagcagccggatctcaAGCGGATTTTTTCGCTTTTTTCTC |
| 1712 | CGTTCCGACCACTAACCCGAC |
| 1713 | GCTGAACTGGCAGGCTTCTCT |
| 1714 | TTCGAAGTAGAAGCGGACCCG |
| M8-2 | pCB17-12 | C. thermocellum AdhE | N-histag | 1184 | tgagatccggctgctaacaaa |
| 1252 | ccggatatagttcctcctttcag |
| 1524 | CATGGTATATCTCCTTCTTAAAGTTAAACAA |
| 1715 | ttttgtttaactttaagaaggagatataccatgcaccaccaccaccaccacgagaacctgtatttccagggtatgACGAAAATAGCGAATAAATACGAAG |
| 1716 | ctcagcttcctttcgggctttgttagcagccggatctcaTTTCTTCGCACCTCCGTAATA |
| 1717 | GTTGTGCCCGGTACTGGCAAT |
| 1571 | TTATAAGCCCACACCCCAGGG |
| 1572 | GTAGCTGACGGTGGATTTGGCC |
| 1573 | GACACCATAATTGCGGTCGGC |

Supplementary File 1B: Protein sequences used in this study

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| Construct name | Sequence |
| **E. coli AdhE in pCB16-1** | MHHHHHHENLYFQGMAVTNVAELNALVERVKKAQREYASFTQEQVDKIFRAAALAAADARIPLAKMAVAESGMGIVEDKVIKNHFASEYIYNAYKDEKTCGVLSEDDTFGTITIAEPIGIICGIVPTTNPTSTAIFKSLISLKTRNAIIFSPHPRAKDATNKAADIVLQAAIAAGAPKDLIGWIDQPSVELSNALMHHPDINLILATGGPGMVKAAYSSGKPAIGVGAGNTPVVIDETADIKRAVASVLMSKTFDNGVICASEQSVVVVDSVYDAVRERFATHGGYLLQGKELKAVQDVILKNGALNAAIVGQPAYKIAELAGFSVPENTKILIGEVTVVDESEPFAHEKLSPTLAMYRAKDFEDAVEKAEKLVAMGGIGHTSCLYTDQDNQPARVSYFGQKMKTARILINTPASQGGIGDLYNFKLAPSLTLGCGSWGGNSISENVGPKHLINKKTVAKRAENMLWHKLPKSIYFRRGSLPIALDEVITDGHKRALIVTDRFLFNNGYADQITSVLKAAGVETEVFFEVEADPTLSIVRKGAELANSFKPDVIIALGGGSPMDAAKIMWVMYEHPETHFEELALRFMDIRKRIYKFPKMGVKAKMIAVTTTSGTGSEVTPFAVVTDDATGQKYPLADYALTPDMAIVDANLVMDMPKSLCAFGGLDAVTHAMEAYVSVLASEFSDGQALQALKLLKEYLPASYHEGSKNPVARERVHSAATIAGIAFANAFLGVCHSMAHKLGSQFHIPHGLANALLICNVIRYNANDNPTKQTAFSQYDRPQARRRYAEIADHLGLSAPGDRTAAKIEKLLAWLETLKAELGIPKSIREAGVQEADFLANVDKLSEDAFDDQCTGANPRYPLISELKQILLDTYYGRDYVEGETAAKKEAAPAKAEKKAKKSA- |
| **C. thermocellum AdhE in pCB17-12** | MHHHHHHENLYFQGMTKIANKYEVIDNVEKLEKALKRLREAQSVYATYTQEQVDKIFFEAAMAANKMRIPLAKMAVEETGMGVVEDKVIKNHYASEYIYNAYKNTKTCGVIEEDPAFGIKKIAEPLGVIAAVIPTTNPTSTAIFKTLIALKTRNAIIISPHPRAKNSTIEAAKIVLEAAVKAGAPEGIIGWIDVPSLELTNLVMREADVILATGGPGLVKAAYSSGKPAIGVGAGNTPAIIDDSADIVLAVNSIIHSKTFDNGMICASEQSVIVLDGVYKEVKKEFEKRGCYFLNEDETEKVRKTIIINGALNAKIVGQKAHTIANLAGFEVPETTKILIGEVTSVDISEEFAHEKLCPVLAMYRAKDFDDALDKAERLVADGGFGHTSSLYIDTVTQKEKLQKFSERMKTCRILVNTPSSQGGIGDLYNFKLAPSLTLGCGSWGGNSVSDNVGVKHLLNIKTVAERRENMLWFRTPEKIYIKRGCLPVALDELKNVMGKKKAFIVTDNFLYNNGYTKPITDKLDEMGIVHKTFFDVSPDPSLASAKAGAAEMLAFQPDTIIAVGGGSAMDAAKIMWVMYEHPEVDFMDMAMRFMDIRKRVYTFPKMGQKAYFIAIPTSAGTGSEVTPFAVITDEKTGIKYPLADYELLPDMAIVDADMMMNAPKGLTAASGIDALTHALEAYVSMLATDYTDSLALRAIKMIFEYLPRAYENGASDPVAREKMANAATIAGMAFANAFLGVCHSMAHKLGAFYHLPHGVANALMINEVIRFNSSEAPTKMGTFPQYDHPRTLERYAEIADYIGLKGKNNEEKVENLIKAIDELKEKVGIRKTIKDYDIDEKEFLDRLDEMVEQAFDDQCTGTNPRYPLMNEIRQMYLNAYYGGAKK- |

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| **Supplementary File 1C: Data collection and processing.** | | | |
|  | **Extended Final** | **Extended Model Building** | **Compact Model Building** |
| **Data collection** |  |  |  |
| Microscope/camera | Krios/Falcon 4 | Talos Arctica/K3 | Krios/Falcon 4 |
| Voltage (kV) | 300 | 200 | 300 |
| Magnification | 96,000 | 45,000 | 96,000 |
| Electron dose (e-/Å2) | 60 | 90 | 60 |
| Pixel Size (Å) | 0.82 | 0.89 | 0.82 |
| Defocus Range (μm) | 1.5-3.0 | 1.2-2.5 | 1.0-3.0 |
| Processing | Single particle analysis | Single particle analysis | Single particle analysis |
| Symmetry imposed | C1 | C1 | C1 |
| Initial particle images (no.) | 504,494 | 352,061 | 959,561 |
| Final particle images (no.) | 90,912 | 103,973 | 64,977 |
| Map resolution (Å)-(FSC threshold model) | 3.28-(0.143) | 3.8-(0.143) | 3.93-(0.143) |
| **Refinement and validation** |  |  |  |
| Map sharpening (B-factor) (Å-2) | -97.02 | -99.00 |  |
| **Model composition** |  |  |  |
| No. of chains | 11 | 10 |  |
| Atmos (no.) | 30,785 | 31,285 |  |
| Residues (no.) | 3,994 | 4,047 |  |
| Ligands (no.) | Fe2+ (5) | Fe2+ (4) |  |
| Bond lengths (Å) | 0.003 | 0.004 |  |
| Bond angles (˚) | 0.590 | 0.633 |  |
| Ramachandran favored % | 95.26 | 93.92 |  |
| Ramachandran allowed % | 4.74 | 6.01 |  |
| Ramachandran outliers % | 0.00 | 0.08 |  |
| Rotamers outliers % | 0.00 | 0.00 |  |
| MolProbity score | 1.65 | 1.92 |  |
| Clashscore | 5.84 | 9.80 |  |
| CC (mask) | 0.86 | 0.79 |  |
| CC (box) | 0.70 | 0.70 |  |
| CC (peaks) | 0.70 | 0.62 |  |
| CC (volume) | 0.82 | 0.78 |  |
| Mean CC for ligands | 0.84 | 0.77 |  |