Supporting Information

Zhu et al. 10.1073/pnas.1316569111

SI Materials and Methods

Cultivation of *Acinetobacter baumannii* ATCC19606 and Mutants. Either a defined medium or Luria broth (LB) was used to cultivate the wild-type or the mutants (Δ*cntA*::*aacC1*, Δ*cntB*::*aacC1*). The defined medium contained NH_4Cl (1 g/L^{-1}), NaCl (0.5 g/L^{-1}), KH_2PO_4 (3 g/L^{-1}), $Na_2HPO_4{\cdot}7H_2O$ (12.8 g/L^{-1}), $MgSO_4{\cdot}7H_2O$ (0.5 g/L^{-1}), $CaCl_2{\cdot}2H_2O$ (0.15 g/L^{-1}), $Na_2MoO_4{\cdot}2H_2O$ (0.5 mg/L^{-1}), $FeCl_3$ (50 μM), and a mix of the following vitamins or supplements, including biotin (0.4 mg/L^{-1}), folic acid (0.4 mg/L^{-1}), pyridoxine hydrochloride (2 mg/L^{-1}), thiamine hydrochloride (1 mg/L^{-1}), riboflavin (1 mg/L^{-1}), nicotinic acid (1 mg/L^{-1}), pantothenic acid (1 mg/L^{-1}), vitamin B_{12} (20 mg/L^{-1}), 4-aminobenzoic acid (1 mg/L^{-1}), and lipoic acid (1 mg/L^{-1}). The LB medium contained tryptone (10 g/L^{-1}), yeast extract (5 g/L^{-1}), and NaCl (5 g/L^{-1}), and the pH was adjusted using NaOH (0.2 μM). Luria agar (LA) was made as LB and agar was added (15 g/L^{-1}).

Growth Curves of *A. baumannii* and the Mutants on Carnitine or Succinate. To characterize growth of the ATCC 19606 wild-type strain (*Salmonella* Genetic Stock Centre, University of Calgary, Calgary, Canada) and the mutants, carnitine, or succinate was added to the defined medium to a final concentration of 20 mM as the sole carbon and energy sources. The wild-type strain and the mutants were cultivated in 5 mL LB for 8 h at 37 °C while shaking (200 rpm). The cultures were then diluted 1:50 to inoculate the defined medium containing 20 mM succinate. These cultures were then incubated at 37 °C for 15–16 h and the pellet was collected by centrifugation at 3,000 × *g* for 10 min. Cell pellets were then washed twice with sterile distilled water and resuspended in 5 mL of water. Finally, 5 mL of the defined medium with either succinate or carnitine was inoculated with 100 μL of washed culture and distributed into eight wells each in a 96-well tissue culture plate (sterile, F-bottom, with lid; Cellstar, Greiner bio-one), which was then incubated at 37 °C and the optical density at 600 nm was recorded every 10 min using a Multiskan GO spectrophotometer (Thermo Scientific).

Cultivation of *A. baumannii* and the Mutants for the Quantification of Trimethylamine and Carnitine by Ion Chromatography. To quantify the production of trimethylamine (TMA) in the wild-type and the mutants, cultures were set up in triplicate in Corning cell culture flasks (surface area 75 cm^2) with 50 mL of the defined medium supplemented with both carnitine (20 mM) and succinate (20 mM). Inoculum was prepared as described above. The flasks were incubated vertically at 37 °C while shaking continuously (200 rpm). A 2-mL sample was taken from each flask at $t = 0$, 4, 8, 12, and 24 h. Optical density at 600 nm was recorded immediately for every sample at each time point. The samples were then passed through 0.22-μm filters to remove microbial cells and the supernatants were diluted 1:10 in double distilled water, which was then quantified for TMA and carnitine using a cation-exchange ion chromatography (Metrohm 881 Compact IC Pro) equipped with a Metrosep C4/250 mm separation column and a conductivity detector (Metrohm).

Marker-Exchange Mutagenesis of *cntA* and *cntB*. To obtain targeted deletion of *cntA* and *cntB* in *A. baumannii* ATCC 19606, we used the mutagenesis strategy developed by van Aartsen and Rajakumar (1), requiring the creation of a suicide plasmid containing the upstream and the downstream flanking regions of the targeted gene separated by a gentamicin antibiotic cassette, followed by transfer of the construct to *A. baumannii*.

Upstream and downstream flanking regions of *cntA* or *cntB* (~1 kb) were amplified from the genomic DNA of ATCC 19606 using primers indicated in Table S5 and the gentamicin-resistant gene cassette (*aacC1*) was amplified from pUC18R6K-mini-Tn*7*T-Gm (2). PCR amplification was performed using Phusion High-Fidelity DNA Polymerase (Thermo Scientific) according to manufacturer's specifications. To join upstream and downstream flanking regions with the gentamicin-resistant gene cassette, spliced overlap extension-PCR (SOE-PCR) was performed (3) using KOD Hot Start DNA Polymerase (Merck). Ten nanograms of each flanking region and the gentamicin-resistant gene cassette were mixed and used as the template. SOE-PCR was run without primers for five cycles and then for 30 cycles after the primers were added (PR2768 and PR2771 for *cntA*; PR2774 and PR2777 for *cntB*) to generate a ~3-kb PCR product. Both SOE-PCR products and the vector (pJTOOL-3) were digested with NotI (New England Biolabs) for 4 h at 37 °C and the bands were purified from an agarose gel. Ligation was performed using T4 DNA ligase (Promega) using 50 ng of digested vector and 2:1 for *cntA* or 4:1 for *cntB* insert:vector molar ratio. Ligation was carried out for 7 h at 15 °C and 10 μL of ligation mix was transformed into chemically competent *Escherichia coli* CC118λ*pir*, which was selected on LA with 30 μg/mL^{-1} chloramphenicol and 15 μg/mL^{-1} gentamicin. Plasmids with insert were selected and tested by digestion to confirm the presence and the orientation of the insert. Finally, selected plasmids were transferred to chemically competent *E. coli* S17-1λ*pir*.

Conjugation was used to efficiently transfer the suicide plasmid to ATCC 19606. The donor *E. coli* strains and the recipient *A. baumannii* strain ATCC 19606 were grown overnight in LB and 100 μL were used to inoculate 10 mL LB. Optical density at 600 nm was measured at regular intervals and growth was suspended when OD_{600} reached 0.3 ~ 0.4. Bacterial culture (5 mL) were collected by centrifugation at 3,000 × *g* for 10 min and washed with 1 mL LB twice and resuspended in 1 mL LB after the final wash. Next, 200 μL of donor strains were mixed with 200 μL of the recipient, centrifuged at 16,000 × *g* for 2 min, resuspended with 100 μL of 10% (wt/vol) sterile glycerol, spread on LA, and incubated at 37 °C for 24 h. Bacterial cultures were collected in 1 mL of 10% (wt/vol) glycerol, serially diluted, and plated on Simmons citrate agar (Oxoid) with 80 μg/mL^{-1} gentamicin. Selected colonies were screened using colony-PCR to detect the insertion of the suicide plasmid at either the upstream flanking region or the downstream flanking region. Selection for double-crossover deletion mutants and the loss of plasmid backbone were carried out by sucrose counter selection because of the presence of a *sacB* gene. Single crossover mutants were cultivated in LB medium overnight and plated out on LA with 6% sucrose, which was subsequently incubated overnight at 37 °C. Screen for double-crossover deletion mutants was carried out by colony PCR. Primers used for screening are listed in Table S5.

Complementation of the Δ*cntA* and Δ*cntB* Mutants of *A. baumannii*. An ~7.8-kb DNA fragment comprising the carnitine oxygenase/reductase gene cluster (Fig. 1*C*) was amplified from the genomic DNA of strain ATCC 19606 using the primers PR3103/PR3104 (Table S5) with a proofreading DNA polymerase (Phusion High-Fidelity DNA polymerase, Thermo Scientific). The PCR product was A-tailed using the GoTaq Polymerase (Promega), purified and cloned into the pGEM-Teasy cloning vector (Promega). The insert was amplified from vector pGEM-Teasy using the primers PR3120/PR3121 with the Phusion High-Fidelity DNA

Polymerase (Thermo Scientific), and the PCR product (30 μg) was subsequently digested with BamHI-HF (New England Biolabs) and KpnI-HF (New England Biolabs) for 4 h at 37 °C. The digested product was run in a 1% (wt/vol) agarose gel and the ∼8-kb band was excised from the gel and purified. The pMQ300 vector (4) was prepared similarly by digesting 5 μg of the vector followed by gel purification. Thirty-five nanograms of the purified pMQ300 vector were ligated with the gel-purified insert in a 1:1 molar ratio for 6 h at 15 °C using the T4 DNA Ligase (Promega). Ten microliters of the ligation product were chemically transformed into the competent cells of *E. coli* DH5α and the colonies were selected on LA supplemented with 140 $\mu g/mL^{-1}$ hygromycin. The resulting plasmid pKR706, containing the carnitine oxygenase/reductase gene cluster was transferred to chemically competent *E. coli* S17-1λ*pir*, which was used as the donor for the conjugation with the Δ*cntA::aacC1* and the Δ*cntB::aacC1* mutant, respectively. Complemented mutants containing pKR706 were selected on LA plates supplemented with 280 $\mu g/mL^{-1}$ hygromycin and 30 $\mu g/mL^{-1}$ chloramphenicol.

Growth of *E. coli* SE11 and *Citrobacter freundii* M3 on Carnitine. To characterize the growth of these strains on carnitine, the bacteria were inoculated to the defined medium as described above and carnitine was added as the sole carbon and energy source (final concentration, 5 mM), and the cultures were incubated at 37 °C while shaking continuously (200 rpm). Optical density at 600 nm was recorded and 2-mL samples were withdrawn from the culture at 0, 4, 8, 12, and 24 h. The culture supernatant was collected by passing through 0.22-μm filters before quantification for TMA and carnitine by cation-exchange ion chromatography, as described above.

Heterologous Overexpression of *cntA*/*cntB*, Site-Directed Mutagenesis and Characterization of the CntA Mutants. The *cntA* and *cntB* genes were amplified from *A. baumannii* and inserted into the expression vector pCOLADuet-1 (Novagen) under the BamHI/HindIII sites or the vector pET28a (Novagen) under the NdeI/HindIII sites. Coexpression of *cntA*/*cntB* was achieved by insertion into pCOLADuet-1 under the BamHI/HindIII and the NdeI/KpnI sites, respectively. The CntA mutants (E205D, E205A) were chemically synthesized by GenScript and inserted into the expression vector pET28a under the NdeI/HindIII sites. The resulting plasmids were then transformed into the expression host *E. coli* BLR(DE3) pLysS (Merck Biosciences). For protein overexpression, *E. coli* cells were grown at 37 °C to an OD_{600} of 0.5, and isopropyl β-D-1-thiogalactopyranoside (IPTG) was then added to a final concentration of 0.2 mM and the induction of protein expression was then carried out at 18 °C before harvesting. Proteins were purified by His-tag affinity purification from recombinant *E. coli* after induction with IPTG according to the manufacturer's protocol (Merck). The activity of the reconstituted enzyme was assayed at room temperature (∼22 °C) by quantifying NADH oxidation and carnitine-dependent TMA production. A 1-mL enzyme assay mixture contained 10 mM Hepes buffer (pH 7.6), 60 μg purified CntA and CntB, respectively, 0.25 mM carnitine, and 0.25 mM NADH. To determine TMA formation from crude extract of recombinant *E. coli* harboring *cntA*, *cntB* or *cntAB*, 500 μg of cell-free crude extracts were used. Coupling efficiency was determined as the ratio of the total amount of TMA formed to the amount of NADH consumed.

Substrate Profile of CntAB. To test the substrate specificity of carnitine oxygenase (CntAB), the enzyme was purified from recombinant *E. coli*. Protein concentrations were quantified by the Bradford method using the Quick Start Bradford Protein Assay Kit (Bio-Rad). The following substrates were purchased from Sigma-Aldrich and tested, including D-, L-carnitine, γ-butyrobetaine, glycine betaine, choline chloride, trimethylamine hydrochloride, and trimethylamine *N*-oxide. Enzyme activity was assayed at room temperature (∼22 °C) by quantifying NADH oxidation. A 1-mL enzyme assay mixture contained 10 mM Hepes buffer (pH 7.6), 60 μg purified CntA and CntB, respectively, 0.25 mM substrate, and 0.25 mM NADH.

Validation of TMA Production from Carnitine by Gas Chromatography-Mass Spectrometry. To further confirm the identity of TMA production from carnitine oxidation by CntAB, the enzyme was purified and the activity was reconstituted in vitro by adding CntA and CntB to the enzyme assay as described above. Controls were set up by using CntA or CntB only. The reaction was initiated by adding NADH. The enzyme assay was then incubated at room temperature (∼22 °C) for 30 min. The reaction was terminated by adding 0.8 g KOH, and TMA produced from carnitine oxidation was subsequently extracted by toluene as described previously (5). TMA identification was carried out using an Agilent 6890/5973 gas chromatography-mass spectrometry (GC-MS) platform equipped with an automatic liquid sampler. An aliquot (1 μL) of toluene layer was injected into GC-MS. GC conditions were as follows: column, Agilent HP-5ms capillary column (30 m X 0.25 mm i.d.; film thickness, 0.25 μm); column temperature, 40 °C for 2.5 min, then the temperature was increased at maximum rate up to 230 °C, followed by 2 min at 230 °C; carrier gas, helium; flow rate, 0.2 mL/min^{-1}; split ratio, 10:1.

Characterization of CntA and the CntA Mutants (E205A, E205D) by Circular Dichroism and Native-PAGE. Purified recombinant CntA and the mutants were dialyzed against 200 mM sodium phosphate (pH 7.0) containing 200 mM NaCl. Circular dichroism (CD) spectra were recorded in the range of 195–260 nm using a Jasco J-815 spectrometer (Jasco) using a quartz cuvette of 1-mm path length at room temperature (∼22 °C). Spectra were collected eight times per sample. Data were expressed as mean residue ellipticity in degrees·cm^2·$dmol^{-1}$. Spectra were deconvoluted using the online program DICHROWEB (http://dichroweb.cryst.bbk.ac.uk/html/home.shtml) (6, 7) and the CDSSTR algorithm was used to estimate the percentages of each secondary structure in CntA and its mutants using the reference protein set 7 (8). Native-PAGE was performed using a NuSep 12% (wt/vol) Tris·glycine precast polyacrylamide gel (NuSep) at a constant voltage (200 V) at 4 °C for 1.5 h with an Invitrogen electrophoresis system. The gels were stained with the Fast Blue reagent (Expedeon). The NativeMark unstained protein standard from Novex was used to estimate the native size of CntA and the mutants.

1. van Aartsen JJ, Rajakumar K (2011) An optimized method for suicide vector-based allelic exchange in *Klebsiella pneumoniae*. *J Microbiol Methods* 86(3):313–319.
2. Choi KH, et al. (2005) A Tn7-based broad-range bacterial cloning and expression system. *Nat Methods* 2(6):443–448.
3. Choi KH, Schweizer HP (2005) An improved method for rapid generation of unmarked *Pseudomonas aeruginosa* deletion mutants. *BMC Microbiol* 5(30):30–41.
4. Kalivoda EJ, et al. (2011) New vector tools with a hygromycin resistance marker for use with opportunistic pathogens. *Mol Biotechnol* 48(1):7–14.
5. daCosta KA, Vrbanac JJ, Zeisel SH (1990) The measurement of dimethylamine, trimethylamine, and trimethylamine N-oxide using capillary gas chromatography-mass spectrometry. *Anal Biochem* 187(2):234–239.
6. Whitmore L, Wallace BA (2004) DICHROWEB, an online server for protein secondary structure analyses from circular dichroism spectroscopic data. *Nucleic Acids Res* 32(Web Server issue):W668–W673.
7. Whitmore L, Wallace BA (2008) Protein secondary structure analyses from circular dichroism spectroscopy: Methods and reference databases. *Biopolymers* 89(5):392–400.
8. Sreerama N, Woody RW (2000) Estimation of protein secondary structure from circular dichroism spectra: Comparison of CONTIN, SELCON, and CDSSTR methods with an expanded reference set. *Anal Biochem* 287(2):252–260.

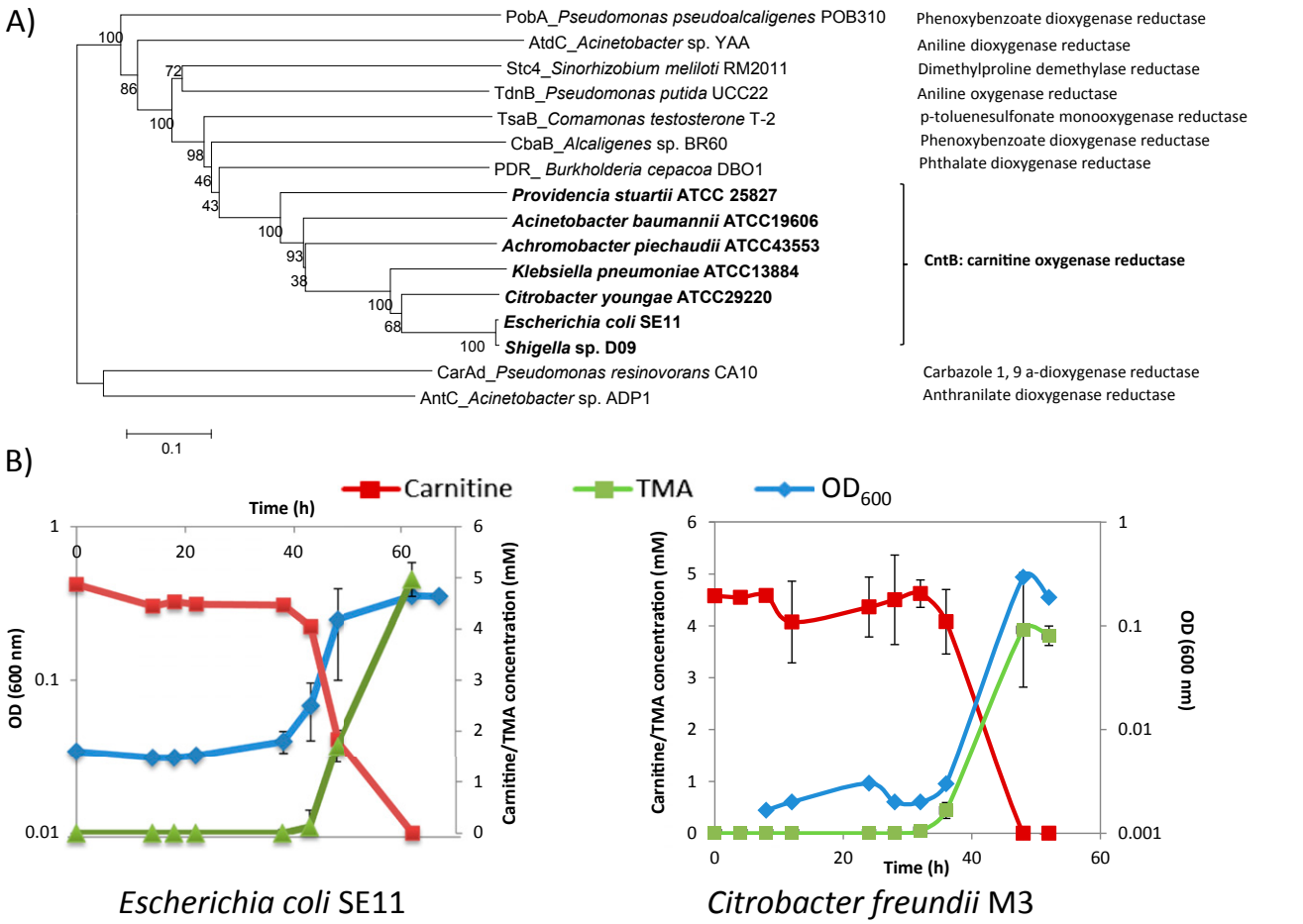


Fig. S1. (*A*) Neighbor joining phylogenetic tree of CntB and other closely related reductase proteins (~320 amino acids). The bar represents one substitution per 10 amino acids. (*B*) Growth of *E. coli* SE11 and *C. freundii* M3 on carnitine as the sole carbon source and production of TMA. Error bars represent SD of measurements from three biological replicates.

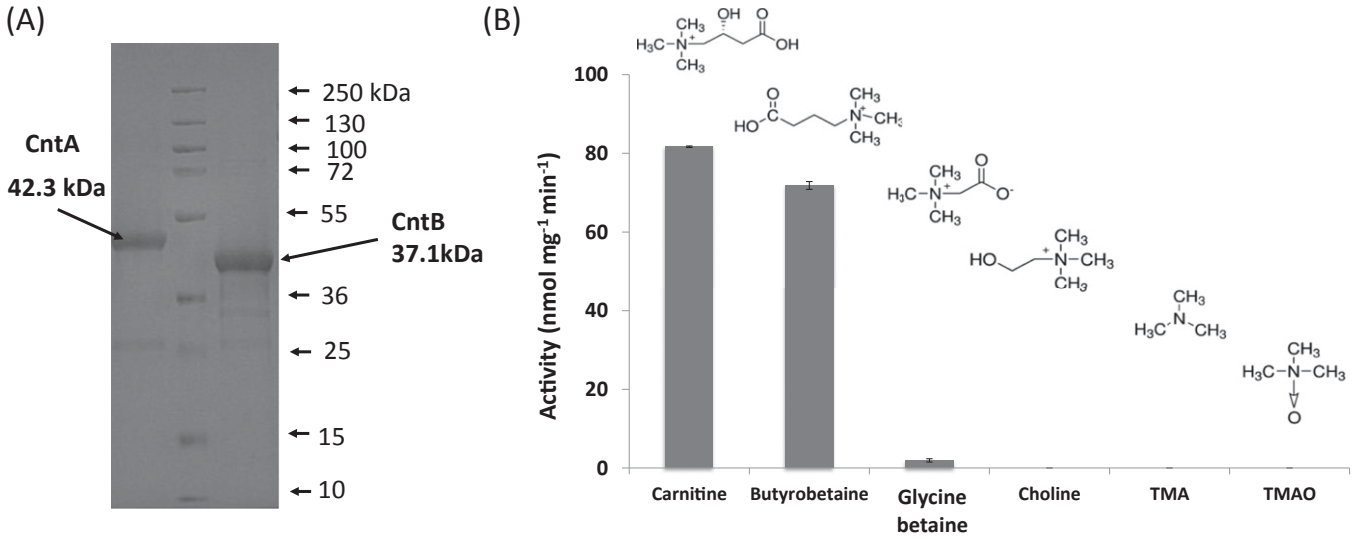


Fig. S2. (*A*) SDS/PAGE analysis of purified CntA and CntB from recombinant *Escherichia coli*. (*B*) Substrate profile of the purified carnitine oxygenase.

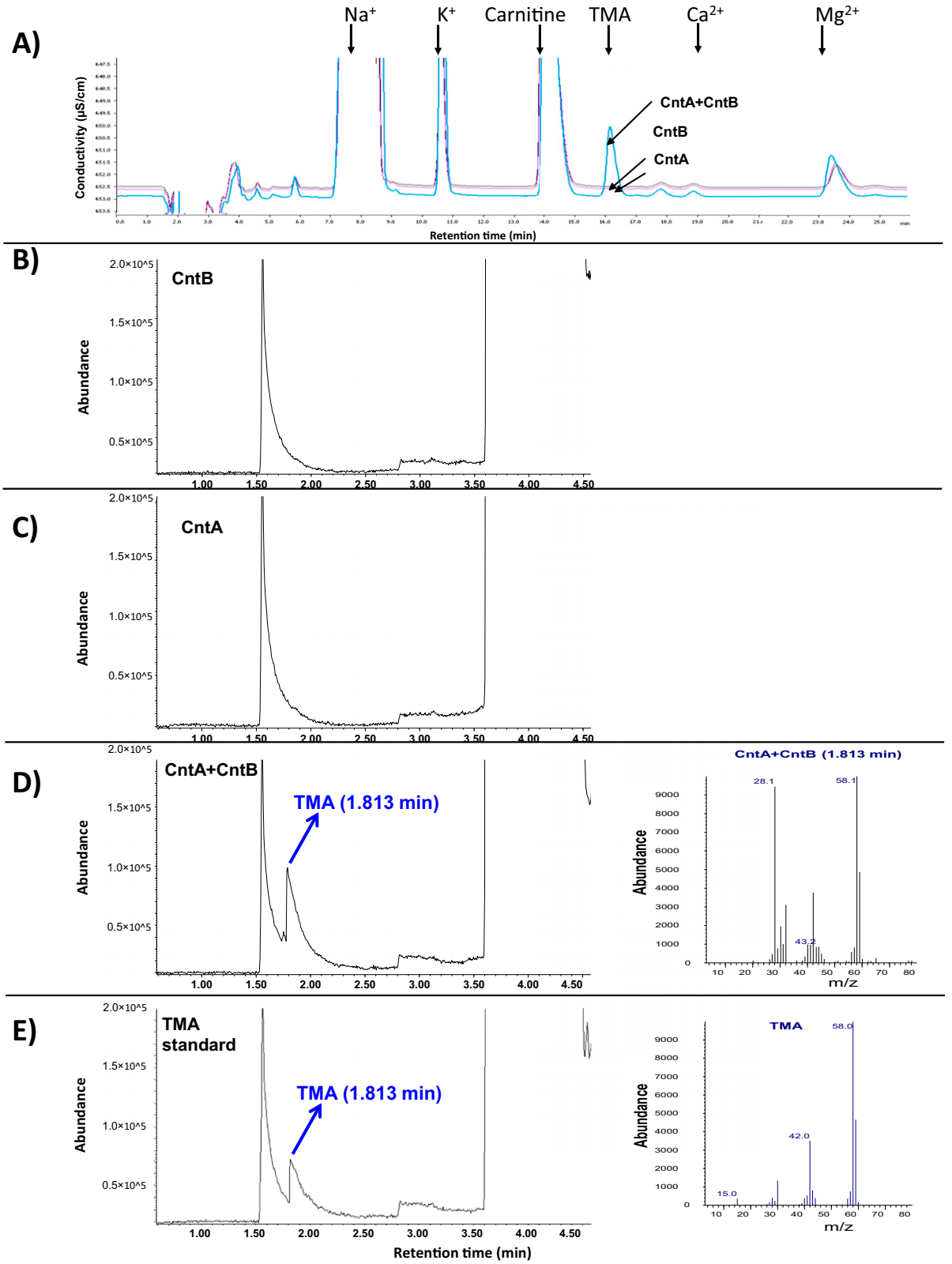


Fig. S3. Ion chromatography quantification (*A*) and GC-MS identification (*B–E*) of TMA production from carnitine using purified recombinant CntA alone, CntB alone or CntA+CntB. Mass spectrum of TMA from carnitine oxidation by CntAB were compared with that of authentic TMA standard (shown on *D*, *Right* and *E*, *Right*, respectively).

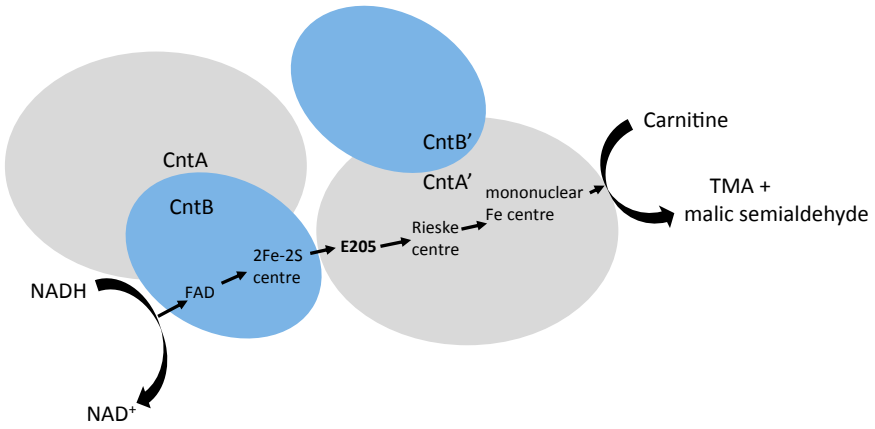
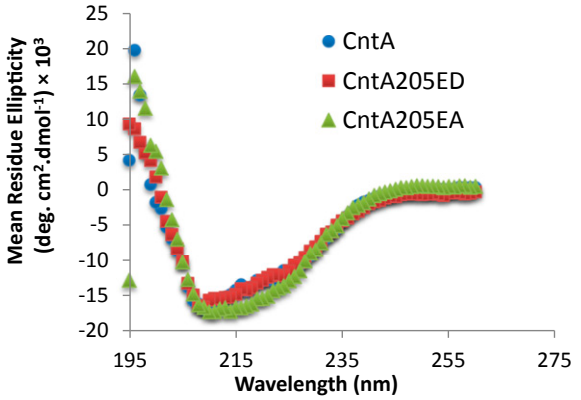


Fig. S4. Proposed electron transfer pathway in CntAB and the role of glutamate 205 in CntA. TMA: trimethylamine; NADH: nicotinamide adenine dinucleotide, reduced.

A)



Result	Helix1	Helix2	Strand1	Strand2	Turns	Unordered	Total
CntA	0.36	0.06	0.18	0.12	0.07	0.21	1
CntA E205A	0.36	0.06	0.17	0.12	0.07	0.21	0.99
CntA E205D	0.38	0.07	0.17	0.11	0.06	0.2	0.99

B)

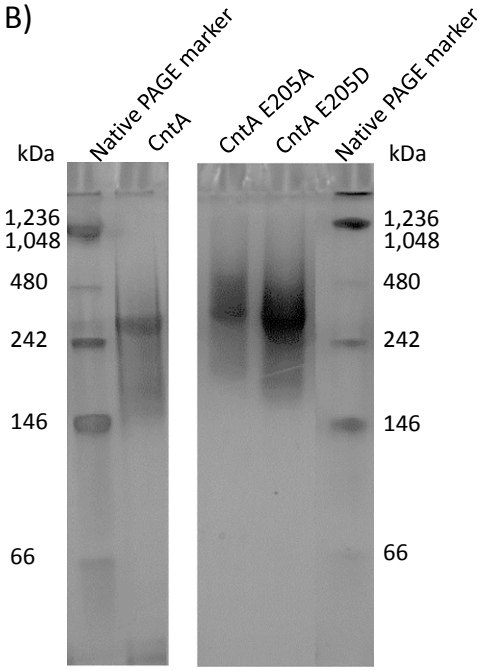


Fig. S5. Characterization of CntA and the site-directed mutants by CD (*A*) and native-PAGE (*B*).

Table S1. CaiT homologs in sequenced Human Microbiome Project (HMP) reference genomes

Locus tag	Annotation	Organism
GCWU000182_02944	Choline/carnitine/betaine transport	*Abiotrophia defectiva* ATCC 49176
HMPREF0004_3863	Conserved hypothetical protein	*Achromobacter piechaudii* ATCC 43553
A60131_010100018802	Putative transporter	*Acinetobacter baumannii* 6013113
A6013_010100011600	Putative transporter	*Acinetobacter baumannii* 6013150
A6014_010100011000	Putative transporter	*Acinetobacter baumannii* 6014059
HMPREF0010_01347	Betaine/choline/glycine transporter	*Acinetobacter baumannii* ATCC 19606
HMPREF0014_03271	Betaine/choline/glycine transporter	*Acinetobacter* sp. RUH2624
HMPREF0013_01187	Conserved hypothetical protein	*Acinetobacter* sp. SH024
HMPREF1705_00723	Osmoprotectant transporter, BCCT family	*Anaerobaculum hydrogeniformans* ATCC BAA-1850
HMPREF1013_04206	OpuD protein	*Bacillus* sp. 2_A_57_CT2
HMPREF1013_03192	Hypothetical protein	*Bacillus* sp. 2_A_57_CT2
HMPREF1013_00505	Glycine betaine transporter	*Bacillus* sp. 2_A_57_CT2
HMPREF1012_02673	OpuD protein	*Bacillus* sp. BT1B_CT2
HMPREF0178_03964	Hypothetical protein	*Bilophila* sp. 4_1_30
HMPREF0179_03321	BCCT family transporter	*Bilophila wadsworthia* 3_1_6
HMPREF0179_00456	BCCT family transporter	*Bilophila wadsworthia* 3_1_6
CAMRE0001_1572	L-carnitine/γ-butyrobetaine antiporter	*Campylobacter rectus* RM3267, CCUG 20446
HMPREF9428_01710	Betaine/carnitine/choline transporter (BCCT) family transporter	*Citrobacter freundii* 4_7_47CFAA
HMPREF9428_02735	L-carnitine/γ-butyrobetaine antiporter	*Citrobacter freundii* 4_7_47CFAA
CSAG_01648	Conserved hypothetical protein	*Citrobacter* sp. 30_2
CSAG_03337	L-carnitine/γ-butyrobetaine antiporter	*Citrobacter* sp. 30_2
CIT292_00938	Choline/carnitine/betaine transport	*Citrobacter youngae* ATCC 29220
CIT292_03024	Choline/carnitine/betaine transport	*Citrobacter youngae* ATCC 29220
Cbac1_010100004187	Putative transporter	*Clostridiales* sp. 1_7_47FAA
CLOSTASPAR_04953	Choline-glycine betaine transporter	*Clostridium asparagiforme* DSM 15981
HMPREF0240_03154	Glycine betaine transporter	*Clostridium* sp. D5
HMPREF0240_03223	Putative osmoprotectant transporter, BCCT family	*Clostridium* sp. D5
HMPREF0240_01437	Osmoprotectant transporter, BCCT family	*Clostridium* sp. D5
HMPREF0322_04106	Transporter, betaine/carnitine/choline family	*Desulfitobacterium hafniense* DP7 (draft 151 contigs)
HMPREF0322_00857	Transporter, betaine/carnitine/choline family	*Desulfitobacterium hafniense* DP7 (draft 151 contigs)
HMPREF0322_00862	Transporter, betaine/carnitine/choline family	*Desulfitobacterium hafniense* DP7 (draft 151 contigs)
HMPREF9457_03501	Hypothetical protein	*Dorea formicigenerans* 4_6_53AFAA
DORFOR_00040	Choline/carnitine/betaine transport	*Dorea formicigenerans* ATCC 27755
EDWATA_03146	L-carnitine/γ-butyrobetaine antiporter	*Edwardsiella tarda* ATCC 23685
EDWATA_03147	L-carnitine/γ-butyrobetaine antiporter	*Edwardsiella tarda* ATCC 23685
HMPREF0864_03292	Betaine/carnitine/choline transporter	*Enterobacteriaceae bacterium* 9_2_54FAA
HMPREF0358_1653	Possible transporter	*Escherichia coli* 83972
HMPREF9345_01358	Transporter, betaine/carnitine/choline transporter family protein	*Escherichia coli* MS 107-1
HMPREF9345_01115	Transporter, betaine/carnitine/choline transporter family protein	*Escherichia coli* MS 107-1
HMPREF9540_03296	Transporter, betaine/carnitine/choline transporter family protein	*Escherichia coli* MS 115-1
HMPREF9540_01454	Transporter, betaine/carnitine/choline transporter family protein	*Escherichia coli* MS 115-1
HMPREF9541_04721	Transporter, betaine/carnitine/choline transporter family protein	*Escherichia coli* MS 116-1
HMPREF9541_02156	Transporter, betaine/carnitine/choline transporter family protein	*Escherichia coli* MS 116-1
HMPREF9346_01791	Transporter, betaine/carnitine/choline transporter family protein	*Escherichia coli* MS 119-7
HMPREF9346_03001	Transporter, betaine/carnitine/choline transporter family protein	*Escherichia coli* MS 119-7
HMPREF9347_04517	Transporter, betaine/carnitine/choline transporter family protein	*Escherichia coli* MS 124-1
HMPREF9347_03685	Transporter, betaine/carnitine/choline transporter family protein	*Escherichia coli* MS 124-1
HMPREF9348_01357	Transporter, betaine/carnitine/choline transporter family protein	*Escherichia coli* MS 145-7
HMPREF9348_03244	Transporter, betaine/carnitine/choline transporter family protein	*Escherichia coli* MS 145-7
HMPREF9543_02303	Transporter, betaine/carnitine/choline transporter family protein	*Escherichia coli* MS 146-1
HMPREF9543_04874	Transporter, betaine/carnitine/choline transporter family protein	*Escherichia coli* MS 146-1
HMPREF9547_02514	Transporter, betaine/carnitine/choline transporter family protein	*Escherichia coli* MS 175-1
HMPREF9547_02765	Transporter, betaine/carnitine/choline transporter family protein	*Escherichia coli* MS 175-1
HMPREF9548_02982	Transporter, betaine/carnitine/choline transporter family protein	*Escherichia coli* MS 182-1
HMPREF9548_02182	Transporter, betaine/carnitine/choline transporter family protein	*Escherichia coli* MS 182-1
HMPREF9549_02457	Transporter, betaine/carnitine/choline transporter family protein	*Escherichia coli* MS 185-1
HMPREF9550_00256	Transporter, betaine/carnitine/choline transporter family protein	*Escherichia coli* MS 187-1
HMPREF9550_01015	Transporter, betaine/carnitine/choline transporter family protein	*Escherichia coli* MS 187-1
HMPREF9551_02675	Transporter, betaine/carnitine/choline transporter family protein	*Escherichia coli* MS 196-1

Table S1. Cont.

Locus tag	Annotation	Organism
HMPREF9551_02516	Transporter, betaine/carnitine/choline transporter family protein	*Escherichia coli* MS 196-1
HMPREF9552_04960	Transporter, betaine/carnitine/choline transporter family protein	*Escherichia coli* MS 198-1
HMPREF9553_02651	Transporter, betaine/carnitine/choline transporter family protein	*Escherichia coli* MS 200-1
HMPREF9530_04260	Transporter, betaine/carnitine/choline transporter family protein	*Escherichia coli* MS 21-1
HMPREF9530_03696	Transporter, betaine/carnitine/choline transporter family protein	*Escherichia coli* MS 21-1
HMPREF9531_02296	Transporter, betaine/carnitine/choline transporter family protein	*Escherichia coli* MS 45-1
HMPREF9534_01299	Transporter, betaine/carnitine/choline transporter family protein	*Escherichia coli* MS 69-1
HMPREF9534_05004	Transporter, betaine/carnitine/choline transporter family protein	*Escherichia coli* MS 69-1
HMPREF9535_02410	Transporter, betaine/carnitine/choline transporter family protein	*Escherichia coli* MS 78-1
HMPREF9535_04534	Transporter, betaine/carnitine/choline transporter family protein	*Escherichia* coli MS 78-1
HMPREF9536_03463	Transporter, betaine/carnitine/choline transporter family protein	*Escherichia coli* MS 84-1
HMPREF9536_02737	Transporter, betaine/carnitine/choline transporter family protein	*Escherichia coli* MS 84-1
ECSF_0045	Putative carnitine transporter	*Escherichia coli* O150:H5 SE15
ECSE_0041	Putative carnitine transporter	*Escherichia coli* SE11
ECSE_1975	Putative transport protein	*Escherichia coli* SE11
ESCG_01223	Choline/carnitine/betaine transporter	*Escherichia* sp. 1_1_43
ESAG_04067	L-carnitine/γ-butyrobetaine antiporter	*Escherichia* sp. 3_2_53FAA
E4_010100004119	Putative transporter	*Escherichia* sp. 4_1_40B
E4_010100018447	L-carnitine/γ-butyrobetaine antiporter	*Escherichia* sp. 4_1_40B
EUBVEN_02747	Choline-glycine betaine transporter	*Eubacterium ventriosum* ATCC 27560
HMPREF0402_02949	Hypothetical protein	*Fusobacterium* sp. 12_1B
HMPREF0454_04935	L-carnitine/γ-butyrobetaine antiporter	*Hafnia alvei* ATCC 51873
HOLDEFILI_01730	Choline/carnitine/betaine transport	*Holdemania filiformis* VPI J1-31B-1, DSM 12042
HMPREF9333_01454	Hypothetical protein	*Johnsonella ignava* ATCC 51276
HMPREF9333_01061	Hypothetical protein	*Johnsonella ignava* ATCC 51276
HMPREF0484_3753	Conserved hypothetical protein	*Klebsiella pneumoniae rhinoscleromatis* ATCC 13884
HMPREF0485_02497	BCCT family betaine/carnitine transporter	*Klebsiella* sp. 1_1_55
HMPREF1024_01931	Betaine/carnitine/choline transporter (BCCT) family transporter	*Klebsiella* sp. 4_1_44FAA
HMPREF9538_04403	Transporter, betaine/carnitine/choline family	*Klebsiella* sp. MS 92-3
HMPREF0490_01850	Hypothetical protein	*Lachnospiraceae bacterium* 6_1_37FAA
HMPREF0987_01668	Hypothetical protein	*Lachnospiraceae bacterium* 9_1_43BFAA
HMPREF0531_2425	BCCT family betaine/carnitine/choline transporter	*Lactobacillus plantarum* ATCC 14917
HMPREF9371_2432	BCCT family osmoprotectant transporter	*Neisseria shayeganii* 871
HMPREF9370_2231	BCCT family osmoprotectant transporter	*Neisseria wadsworthii* 9715
POTG_03408	Choline/carnitine/betaine transporter	*Paenibacillus* sp. D14
HMPREF9024_00300	BCCT family betaine/carnitine transporter	*Pediococcus acidilactici* 7_4
HMPREF0623_1089	Glycine betaine/carnitine/choline transporter	*Pediococcus acidilactici* DSM 20284
HMPREF0693_2800	BCCT family betaine/carnitine/choline transporter	*Proteus mirabilis* ATCC 29906
PROPEN_01218	Choline/carnitine/betaine transport	*Proteus penneri* ATCC 35198
PROVRETT_04514	Choline/carnitine/betaine transport	*Providencia rettgeri* DSM 1131
HMPREF9373_0457	BCCT family betaine transporter	*Psychrobacter* sp. 1501
HMPREF9373_1383	BCCT family betaine/carnitine/choline transporter	*Psychrobacter* sp. 1501
HMPREF7215_2461	Glycine betaine transporter OpuD	*Pyramidobacter piscolens* W5455
ShiD9_010100014456	L-carnitine/γ-butyrobetaine antiporter	*Shigella* sp. D9
ShiD9_010100003772	Putative transporter	*Shigella* sp. D9
HMPREF9372_1646	BCCT family osmoprotectant transporter	*Sporosarcina newyorkensis* 2681
HMPREF0782_2136	BCCT family osmoprotectant transporter	*Staphylococcus aureus aureus* ATCC 51811
HMPREF0783_0859	BCCT family osmoprotectant transporter	*Staphylococcus aureus aureus* ATCC BAA-39
HMPREF0769_10535	BCCT family osmoprotectant transporter	*Staphylococcus aureus aureus* MN8
HMPREF0774_1421	Choline transporter	*Staphylococcus aureus aureus* TCH130
HMPREF0772_10576	BCCT family osmoprotectant transporter	*Staphylococcus aureus aureus* TCH60
HMPREF0773_0708	Choline transporter	*Staphylococcus aureus aureus* TCH70
Sauraur_010100002150	BCCT family betaine/carnitine/choline transporter	*Staphylococcus aureus aureus* USA300_TCH959
HMPREF0786_01385	Osmoprotectant transporter, BCCT family	*Staphylococcus caprae* C87
HMPREF0789_1995	BCCT family betaine/carnitine/choline transporter	*Staphylococcus epidermidis* BCM-HMP0060
HMPREF0793_0722	BCCT family betaine/carnitine/choline transporter	*Staphylococcus epidermidis* M23864:W1
HMPREF0794_1239	BCCT family osmoprotectant transporter	*Staphylococcus epidermidis* M23864:W2(gray)

Table S1. Cont.

Locus tag	Annotation	Organism
HMPREF0797_0715	Transporter, betaine/carnitine/choline transporter (BCCT) family protein	*Staphylococcus epidermidis* SK135
HMPREF0791_2152	BCCT family betaine/carnitine/choline transporter	*Staphylococcus epidermidis* W23144
HMPREF0798_01170	Osmoprotectant transporter, BCCT family	*Staphylococcus hominis hominis* C80
STAHO0001_0325	Choline-glycine betaine transporter	*Staphylococcus hominis* SK119
HMPREF0790_1708	BCCT family osmoprotectant transporter	*Staphylococcus lugdunensis* M23590
STAWA0001_0151	Osmoprotectant transporter, bcct family	*Staphylococcus warneri* L37603, SK66

In the JGI/IMG-HMP database as of February 2013, 754 HMP reference genomes were available; 122 CaiT homologs were found ($E \leq -50$) in 91 unique genomes.

Table S2. Putative *cntA* gene in sequenced HMP reference genomes

Locus tag	Annotation	Organism	Sources of isolation
HMPREF0004_3864	Conserved hypothetical protein	*Achromobacter piechaudii* ATCC 43553	Nose wound, *Homo sapiens*, France
A60131_010100018812	Rieske [2Fe-2S] domain protein	*Acinetobacter baumannii* 6013113	Human skin
A6013_010100011610	Rieske [2Fe-2S] domain protein	*Acinetobacter baumannii* 6013150	Human skin
A6014_010100011010	Rieske [2Fe-2S] domain protein	*Acinetobacter baumannii* 6014059	Human skin
HMPREF0010_01349	Dioxygenase α-subunit	*Acinetobacter baumannii* ATCC 19606	Human urine
HMPREF0014_03273	Dioxygenase α-subunit	*Acinetobacter* sp. RUH2624	Human skin
HMPREF0013_01185	Conserved hypothetical protein	*Acinetobacter* sp. SH024	Human skin
HMPREF9428_01711	Putative dioxygenase subunit α *yeaW*	*Citrobacter freundii* 4_7_47CFAA	Human sigmoid colon
CSAG_01649	Rieske domain-containing protein	*Citrobacter* sp. 30_2	Human feces
CIT292_03025	Ring-hydroxylating dioxygenases, large terminal subunit	*Citrobacter youngae* ATCC 29220	Human feces
HMPREF9345_01357	Rieske [2Fe-2S] domain protein	*Escherichia coli* MS 107-1	Human gastrointestinal tract
HMPREF9540_01453	Rieske [2Fe-2S] domain protein	*Escherichia coli* MS 115-1	Human intestinal tract
HMPREF9541_04722	Rieske [2Fe-2S] domain protein	*Escherichia coli* MS 116-1	Human gastrointestinal tract
HMPREF9346_03000	Rieske [2Fe-2S] domain protein	*Escherichia coli* MS 119-7	Human gastrointestinal tract
HMPREF9347_04518	Rieske [2Fe-2S] domain protein	*Escherichia coli* MS 124-1	Human gastrointestinal tract
HMPREF9348_01358	Rieske [2Fe-2S] domain protein	*Escherichia coli* MS 145-7	Human gastrointestinal tract
HMPREF9543_02302	Rieske [2Fe-2S] domain protein	*Escherichia coli* MS 146-1	Human gastrointestinal tract
HMPREF9547_02513	Rieske [2Fe-2S] domain protein	*Escherichia coli* MS 175-1	Human gastrointestinal tract
HMPREF9548_02983	Rieske [2Fe-2S] domain protein	*Escherichia coli* MS 182-1	Human gastrointestinal tract
HMPREF9550_00257	Rieske [2Fe-2S] domain protein	*Escherichia coli* MS 187-1	Human gastrointestinal tract
HMPREF9551_02674	Rieske [2Fe-2S] domain protein	*Escherichia coli* MS 196-1	Human gastrointestinal tract
HMPREF9552_03168	Rieske [2Fe-2S] domain protein	*Escherichia coli* MS 198-1	Human gastrointestinal tract
HMPREF9530_03695	Rieske [2Fe-2S] domain protein	*Escherichia coli* MS 21-1	Human gastrointestinal tract
HMPREF9534_05005	Rieske [2Fe-2S] domain protein	*Escherichia coli* MS 69-1	Human gastrointestinal tract
HMPREF9535_02411	Rieske [2Fe-2S] domain protein	*Escherichia coli* MS 78-1	Human gastrointestinal tract
HMPREF9536_02738	Rieske [2Fe-2S] domain protein	*Escherichia coli* MS 84-1	Human gastrointestinal tract
ECSE_1976	Putative dioxygenase α subunit	*Escherichia coli* SE11	Human gastrointestinal tract
E4_010100004114	Predicted 2Fe-2S containing protein	*Escherichia* sp. 4_1_40B	Human gastrointestinal tract
HMPREF0484_3754	Conserved hypothetical protein	*Klebsiella pneumoniae rhinoscleromatis* ATCC 13884	Human airways sample
HMPREF0485_02496	Dioxygenase subunit α *yeaW*	*Klebsiella* sp. 1_1_55	Human gastrointestinal tract
HMPREF1024_01930	Hypothetical protein	*Klebsiella* sp. 4_1_44FAA	Human gastrointestinal tract
HMPREF9538_04404	Rieske [2Fe-2S] domain protein	*Klebsiella* sp. MS 92-3	Human gastrointestinal tract
PROVRETT_04639	Ring-hydroxylating dioxygenases, large terminal subunit	*Providencia rettgeri* DSM 1131	Human feces
PstuA_020100020983	Dioxygenase, α-subunit	*Providencia stuartii* ATCC 25827	Human gastrointestinal tract
SBO_1286	Hypothetical protein	*Shigella boydii* Sb227	Epidemic in China in 1950s
SDY_1706	Hypothetical protein	*Shigella dysenteriae* Sd197	Epidemic in China in 1950s
SSON_1359	Hypothetical protein	*Shigella sonnei* Ss046	Epidemic in China in 1950s
ShiD9_010100003767	Predicted 2Fe-2S containing protein	*Shigella* sp. D9	Human gastrointestinal tract
HMPREF9372_1747	Rieske [2Fe-2S] domain protein	*Sporosarcina newyorkensis* 2681	Human blood

In the JGI/IMG-HMP database as of February 2013, 754 HMP reference genomes were available; 39 CntA homologs ($E \leq -50$, query sequence: CntA of *A. baumannii* ATCC19606) were found in 39 unique genomes.

Table S3. Rieske-type proteins used in CntA phylogenetic analyses

GenBank or PDB accession no.	Gene	Enzyme	Organism
M64747	*xylX*	Toluate 1,2-dioxygenase	*Pseudomonas putida*
AF071556	*antA*	Anthranilate dioxygenase	*Acinetobacter* sp. ADP1
AF119621	*ditA*	Abietane diterpenoids oxygenase	*Pseudomonas abietaniphila*
AJ223220	*dxnA*	Dioxin dioxygenase	*Sphingomonas* sp. RW1
2B1X	*nahAc*	Naphthalene 1,2-dioxygenase	*Rhodococcus* sp. NCIMB12038
2XR8	*bhpAE*	Biphenyl dioxygenase	*Burkholderia xenovorans* Lb400
1WQL	*cumA*	Cumene dioxygenase	*Pseudomonas fluorescens* Ip01
3EN1	*todC1*	Toluene 2,3-dioxygenase	*Pseudomonas putida*
2GBW	*bphA*	Biphenyl 2,3-dioxygenase	*Sphingomonas yanoikuyae* B1
2CKF	*pahAc*	PAH dioxygenase	*Sphingomonas* sp. Chy-1
2BMO	*nbzAC*	Nitrobenzene dioxygenase	*Comamonas* sp. JS765
1O7W	*nahAc*	Naphthalene 1,2-dioxygenase	*Pseudomonas putida*
2ZYL	*kshA*	3-ketosteroid-9-α-hydroxylase	*Mycobacterium tuberculosis*
1Z02	*oxoO*	2-oxoquinoline 8-monooxygenase	*Pseudomonas putida*
1WW9	*carA*	Carbazole 1, 9 a-dioxygenase	*Janthinobacterium* sp. J3
3GKE	*dmo*	Dicamba monooxygenase	*Stenotrophomonas maltophilia*
Q44256	*cbaA*	3-chlorobenzoate-3,4-dioxygenase	*Comamonas testosteroni*
3VCP	*stc2*	Dimethylproline demethylase	*Sinorhizobium meliloti* RM2011
AAG08795	*gbcA*	Glycine betaine demethylase	*Pseudomonas aeruginosa*
U85780	*cmo*	Choline monooxygenase	*Spinacia oleracea*
AB303389	*cmo*	Choline monooxygenase	*Amaranthus tricolor*
CAE17671	*cmo*	Choline monooxygenase	*Oryza sativa*

Table S4. Bacterial strains and plasmids used in this study

Strains and plasmids	Description, genotype, relevant characteristics	Source
Acinetobacter baumannii		
ATCC 19606		University of Leicester laboratory collection
Δ*cntA*::*aacC1*	ATCC 19606 derivative with *cntA* deletion	Present study
Δ*cntB*::*aacC1*	ATCC 19606 derivative with *cntB* deletion	Present study
Escherichia coli		
JM109	General cloning	Promega
BLR(DE3)pLysS	Heterologous expression of *cntA*/*cntB* under the T7 promoter	Novagen
CC118λ*pir*	Δ(*ara-leu*) *araD* _*lacX74 galE galK phoA thi-1 rpsE rpoB argE*(Am) *recA1*; lysogenized with λ*pir* phage	Simon et al. (1)
S17-1λ*pir*	*hsdR recA pro* RP4-2 (Tc::Mu; K_m::Tn*7*)(λ*pir*)	Simon et al. (1)
Plasmids		
pET28a	For overexpression of *cntA* and its mutants (E205D, E205A)	Novagen
pCOLADuet-1	For coexpression of *cntA* and *cntB*	Novagen
pJTOOL-3	Suicide vector for *Acinetobacter baumannii*, R6K *ori mob*RP4 *sacB*; Cm^R	van Aartsen and Rajakumar (2)
pUC18R6K-mini-Tn*7*T-Gm	Source of gentamicin cassette	Choi et al. (3)
pKR609	pJTOOL-3::Δ*cntA*-GM 5	Present study
pKR612	pJTOOL-3::Δ*cntB*-GM 22	Present study
pMQ300	For complementation of Δ*cntA* and Δ*cntB* mutants; Hph^R; pBBR1 *ori*, *oriT*, URA3 marker	Kalivoda et al. (4)
pKR706	The 7.8 kb carnitine oxygenase gene cluster cloned into the BamHI/KpnI sites of pMQ300	Present study

1. Simon R, Prifer U, Pühler A (1983) A broad host range mobilization system for *in vivo* genetic engineering: Transposon mutagenesis in gram negative bacteria. *Bio/Technology* 1(9):784–791.
2. van Aartsen JJ, Rajakumar K (2011) An optimized method for suicide vector-based allelic exchange in *Klebsiella pneumoniae*. *J Microbiol Methods* 86(3):313–319.
3. Choi KH, et al. (2005) A Tn*7*-based broad-range bacterial cloning and expression system. *Nat Methods* 2(6):443–448.
4. Kalivoda EJ, et al. (2011) New vector tools with a hygromycin resistance marker for use with opportunistic pathogens. *Mol Biotechnol* 48(1):7–14.

Table S5. Primers used in this study

Primer	Sequence (5′–3′)	Note
cntAF	GGATCCGATGAGTGCAGTTGAAAAATTACCTGAAGA	Overexpression of CntA
cntAR	ATGATGAAAGCTTTTATTGATGGTACTGCGCCACAAGAT	
cntBF	GGATCCGATGGCGAGTCATTATGAAATGTT	Overexpression of CntB
cntBR	AAGCTTTTAAAGATCTAATATCAATTTTTTGCC	
cntBF_Co	CATATGGCGAGTCATTATGAAATGTT	Coexpression of CntA and CntB in pCOLADuet-1
cntBR_Co	GGTACCTTAAAGATCTAATATCAATTTTTTGCC	
PR351	CGAATTAGCTTCAAAAGCGCTCTGA	Amplification of the gentamicin cassette (*aacC1*)
PR2773	AATTGGGGATCTTGAAGTTCCT	
PR2768	GCGCGGCCGCGCAGCATTTATTCACCAGCA	Amplification of *cntA* upstream flanking region
PR2769	TCAGAGCGCTTTTGAAGCTAATTCGTTCAACTGCACTCATGTTTTTACTCC	
PR2770	AGGAACTTCAAGATCCCCAATTGCGCAGTACCATCAATAAAAAC	Amplification of *cntA* downstream flanking region
PR2771	GCGCGGCCGCACAGGTCTGGCCTGCATTAC	
PR2774	GCGCGGCCGCGCTTGCAGAAAAGGCAGGTAA	Amplification of *cntB* upstream flanking region
PR2775	TCAGAGCGCTTTTGAAGCTAATTCGATGACTCGCCATATTAGAATC	
PR2776	AGGAACTTCAAGATCCCCAATTTTGGTATTAGATCTTTAATATA	Amplification of *cntB* downstream flanking region
PR2777	GCGCGGCCGCCGAAATGTGGCTTTCCAAGT	
PR2767	TGCAAGCTCAATCAAACTGG	Detection of crossover at *cntA* upstream flanking region
PR319	CTCCGAACTCACGACCGA	
PR2772	GCCAATTTGTACGCCATCTT	Detection of crossover at *cntA* downstream flanking region
PR318	GACATAAGCCTGTTCGGTT	
PR2778	CGAAGAAGGCAAGCGTATTT	Detection of crossover at *cntB* upstream flanking region
PR319	CTCCGAACTCACGACCGA	
PR2764	TTTCCGGCAATTTATTCAGC	Detection of crossover at *cntB* downstream flanking region
PR318	GACATAAGCCTGTTCGGTT	
PR3103	TGCCATCTTCTGTCAGGCTA	Amplification of the 7.8 kb carnitine oxygenase gene cluster from ATCC 19606
PR3104	TATGCCCACCAAATGAACAA	
PR3120	CGGCGGTACCTGTAAAACGACGGCCAGT	Amplification of the carnitine oxygenase gene cluster from pGEM-Teasy
PR3121	CGGCGGATCCATTTAGGTGACACTATAGAAT	