**Clostridium sporogenes** uses reductive Stickland metabolism in the gut to generate ATP and produce circulating metabolites

Yuanyuan Liu¹, Haoqing Chen¹, William Van Treuren², Bi-Huei Hou¹, Steven K. Higginbottom² and Dylan Dodd¹,²

Gut bacteria face a key problem in how they capture enough energy to sustain their growth and physiology. The gut bacterium *Clostridium sporogenes* obtains its energy by utilizing amino acids in pairs, coupling the oxidation of one to the reduction of another—the Stickland reaction. Oxidative pathways produce ATP via substrate-level phosphorylation, whereas reductive pathways are thought to balance redox. In the present study, we investigated whether these reductive pathways are also linked to energy generation and the production of microbial metabolites that may circulate and impact host physiology. Using metabolomics, we find that, during growth in vitro, *C. sporogenes* produces 15 metabolites, 13 of which are present in the gut of *C. sporogenes*-colonized mice. Four of these compounds are reductive Stickland metabolites that circulate in the blood of gnotobiotic mice and are also detected in plasma from healthy humans. Gene clusters for reductive Stickland pathways suggest involvement of electron transfer proteins, and experiments in vitro demonstrate that reductive metabolism is coupled to ATP formation and not just redox balance. Genetic analysis points to the broadly conserved Rnf complex as a key coupling site for energy transduction. Rnf complex mutants show aberrant amino acid metabolism in a defined medium and are attenuated for growth in the mouse gut, demonstrating a role of the Rnf complex in Stickland metabolism and gut colonization. Our findings reveal that the production of circulating metabolites by a commensal bacterium within the host gut is linked to an ATP-yielding redox process.

Proteolytic clostridia are a group of anaerobic bacteria that have the unique ability to grow with amino acids and peptides as their sole energy source. These microbes colonize the gastrointestinal tract of mammals where their metabolic end-products accumulate within the gut and are absorbed into the circulation, mediating important effects on host health. Amino acid metabolites also facilitate syntrophic metabolic interactions among microbes within the gut, with aryl and branched-chain fatty acids being required growth factors for several groups of anaerobic gut bacteria. Despite their importance to human health and disease, we know very little about the metabolic processes that gut bacteria employ to produce such molecules.

It has long been known that *Clostridium sporogenes* obtains its energy by coupling the oxidation of one amino acid to the reduction of another—the Stickland reaction. Oxidative metabolism yields ATP via substrate-level phosphorylation, whereas reductive metabolism is thought to balance redox within the cell. However, several lines of evidence suggest that these reductive pathways may also be linked to energy conservation. First, proline reduction in *C. sporogenes* results in vectorial proton ejection which may energize the cell. Second, reduction of cinnamic acid, a plant secondary metabolite, is linked to ATP formation in *C. sporogenes*. Third, the gene cluster for reductive aromatic amino acid metabolism in *C. sporogenes* carries a bifurcating acyl-CoA dehydrogenase, suggesting that this pathway may also be linked to energy formation involving the *Rhodobacter* nitrogenase-like (Rnf) complex.

Although data suggest that microbial metabolites produced within the gut and that circulate in the host might arise from energy-forming catabolic pathways, experimental evidence is lacking. In the present study, we sought to address the following outstanding questions: (1) What is the extent to which amino acid pairs are used to stimulate the growth of *C. sporogenes*? (2) Which *C. sporogenes* metabolites are produced in the gut and circulate in the host? (3) Is reductive Stickland metabolism linked to ATP formation? (4) What is the role of the Rnf complex in amino acid metabolism and in vivo fitness in the gut?

**Results**

*C. sporogenes* produces 15 metabolites in vitro. *C. sporogenes* ATCC (American Type Culture Collection) 15579 is a proteolytic anaerobic spore-forming bacterium isolated from human faeces. It is genetically tractable, making it an ideal candidate for understanding how amino acid metabolism in the gut contributes to metabolites in the host. When cultured anaerobically in defined medium without carbohydrates, *C. sporogenes* grows rapidly, achieving a doubling time of ~45 min (Fig. 1a). After 24 h of growth, most amino acids are depleted (Fig. 1b and Supplementary Table 1) and 15 different metabolites accumulate in culture supernatants (Fig. 1c).

These metabolites include short-chain fatty acids, branched-chain fatty acids, aromatic fatty acids and amines (Fig. 1c). These metabolites are known to accumulate in the human gut and are not known to be produced by host biochemical pathways at appreciable levels.

*C. sporogenes* uses amino acids in ‘Stickland’ pairs. Early studies with *C. sporogenes* revealed that it metabolizes amino acids in pairs, coupling the oxidation of one with the reduction of another via the...
Stickland reaction. Several amino acid pairs are known to stimulate its growth including Arg/Ile, Ser/Pro, Val/Pro, Leu/Pro and Ile/Pro. However, a comprehensive analysis of the growth-promoting Stickland pairs has not been performed. To evaluate which amino acid pairs are used to promote growth, we performed a high-throughput, growth-based assay where substrates were added in amino acid pairs to promote growth, we performed a Stickland pairs has not been performed. To evaluate which improves growth yields of glucose. In this context, our observation with several amino acids in a pattern similar to that for glucose (Fig. 2a). Serine is deaminated in a single reaction to form pyruvate, a central metabolic intermediate that can be oxidatively decarboxylated, yielding ATP via substrate-level phosphorylation. In our assay, the Ser/Arg pair promoted the largest growth yield of any combination (Fig. 2a). The Ser/Arg combination reached a maximum OD approximately threefold higher than that of either of the two substrates alone (Fig. 2b,c). The Ser/Arg combination did not require supplemental acetate (Fig. 2a versus Fig. 2d), probably reflecting the capacity of serine to satisfy anabolic reactions via pyruvate. Despite the observation that most bacteria carry a copy of serine dehydratase, relatively little is known about its role in metabolism. In asaccharolytic Campylobacter jejuni, serine is preferentially utilized as a growth substrate and a serine dehydratase mutant is attenuated for colonization in the avian gut. Our results provide additional insights into the role of serine in bacterial metabolism, suggesting that it is an important oxidative substrate for Stickland metabolism.

Acetate supplementation had a dramatic impact on certain amino acid pairs. Among the top ten Stickland pairs that were stimulated by addition of acetate, eight included combinations of branched-chain amino acids (serving as reductants) with proline or amino acids that converge on proline (serving as oxidants) (Fig. 2e, red bars). This suggests that neither branched-chain amino acids nor proline and related amino acids such as trans-4-hydroxyproline can satisfy anabolic reactions within the cell.

C. sporogenes amino acid metabolites circulate in the host. To evaluate how C. sporogenes contributes to metabolites in the host, we colonized germ-free mice with C. sporogenes and quantified metabolites in faeces, caecal contents, plasma and urine using stable isotope-dilution liquid chromatography–mass spectrometry (LC–MS) (Supplementary Tables 2–5). Of the 15 metabolites detected in cultures of C. sporogenes (Fig. 1c), 13 were present in the faeces of C. sporogenes-colonized mice 1 week after colonization (Fig. 3a),
indicating that *C. sporogenes* produces these metabolites in vivo. In plasma, four metabolites were elevated in colonized versus germ-free mice, including 5-aminovalerate (5-AVA), phenylpropionate (PPA), 3-(4-hydroxyphenyl)propionate (4-OH-PPA) and indolepropionate (IPA) (Fig. 3a). In *C. sporogenes* mono-colonized mice before antibiotic treatment (Fig. 3b), mean plasma concentrations

**Fig. 2 | Growth-based assay reveals coupled amino acid metabolism in *C. sporogenes*.**

- **a**, *C. sporogenes* grown in basal medium containing ten essential amino acids (glycine, valine, leucine, isoleucine, histidine, methionine, arginine, phenylalanine, tyrosine, tryptophan; 1mM each) supplemented with acetate (40mM) and indicated substrates (25mM) for 48h, recording the maximum OD600 using a microplate spectrophotometer.
- **b**, *C. sporogenes* grown in basal medium containing ten essential amino acids supplemented with acetate and indicated substrates (25mM), monitoring OD hourly for 13h using a spectrophotometer.
- **c**, Maximum ODs from growth in basal medium subtracted from maximum ODs from growth with indicated substrates in **b**.
- **d**, Experiments were performed as in **a**, except that no supplemental acetate was provided.
- **e**, Effect of acetate supplementation on maximum growth yields from substrate combinations. The change in maximum OD represents the maximum OD during growth without acetate subtracted from the maximum OD during growth with acetate. The top ten pairs of substrates where acetate promoted growth are shown in red and the top ten pairs of substrates where acetate diminished growth are shown in blue. In **a**, the growth screen was performed twice independently with technical duplicates. In **d**, the growth screen was performed once with technical duplicates. For **b** and **c**, experiments were repeated independently three times. For **a, b, d** and **e**, representative data are shown. For **c**, data are plotted as means ± s.d. from n = 3 experiments.
Colonized M. musculus, male mice (T ac:SW) aged 8–11 weeks were used for the experiments. For a and b, female mice (T ac:SW) aged 10–12 weeks were used for the experiments. Means ± s.d. from n = 9 mice per group (WT, prdF M. musculus). For c, means ± s.d. from n = 5 mice per group. For d, data are plotted as means ± s.d. from n = 5 mice per group per culture supernatant. For e, data are plotted as means ± s.d. from n = 3 cultures (prdF, fldC, acdA) or n = 6 cultures (WT). For f, data are plotted as means ± s.d. from n = 8 mice per group (WT, prdF, acdA) or n = 9 mice per group (fldC). For a and c, male mice (M. musculus (Tac:SW)) aged 10–12 weeks were used for the experiments. For f, male mice (M. musculus (Tac:SW)) aged 8–11 weeks were used for the experiments.

Fig. 3 | Microbial metabolism within the gut yields metabolites that circulate within the host. a, Faecal and plasma levels of metabolites in germ-free and C. sporogenes-colonized mice. Black arrows indicate metabolites enriched in plasma of C. sporogenes-colonized mice compared with germ-free mice. b, Experimental design for detecting metabolites in circulation of gnotobiotic mice before and after antibiotic treatment. Red arrows indicate timing of plasma collection. c, Quantification of 5-AVA, PPA, 4-OH-PPA and IPA in plasma of gnotobiotic mice. Abx, antibiotic treated; Cs, C. sporogenes-colonized; GF, germ free. d, Overview of pathways for the production of 5-AVA, PPA, 4-OH-PPA and IPA. PrdF is a proline racemase involved in the interconversion of L- and D-proline. FldBC is a phenyl-lactoyl-CoA dehydratase and AcdA an acyl-CoA dehydrogenase. e, Quantification of 5-AVA, PPA, 4-OH-PPA and IPA in 48-h culture supernatants of WT C. sporogenes or prdF, fldC or acdA mutants grown in defined medium (SACC-20 amino acids). f, Quantification of 5-AVA, PPA, 4-OH-PPA and IPA in plasma of gnotobiotic mice 2 weeks after colonization with either WT C. sporogenes or its prdF, fldC or acdA mutants. g, Quantification of 5-AVA, PPA, 4-OH-PPA and IPA in plasma of healthy human blood donors (n = 99). The numbers above plots indicate the total number of individuals with detectable metabolite levels. The red dots indicate concentrations within the accurate measurable range of the assay and the grey dots indicate those that are detected but below the lower limit of quantification. For a, data represent means from n = 5 mice per group. For c, data are plotted as means ± s.d. from n = 5 mice per group. For d, data are plotted as means ± s.d. from n = 3 cultures (prdF, fldC, acdA) or n = 6 cultures (WT). For f, data are plotted as means ± s.d. from n = 8 mice per group (WT, prdF, acdA) or n = 9 mice per group (fldC). For a and c, female mice (M. musculus (Tac:SW)) aged 10–12 weeks were used for the experiments. For f, male mice (M. musculus (Tac:SW)) aged 8–11 weeks were used for the experiments.

Ranged from ~3 μM for PPA to ~100 μM for IPA (Fig. 3c). After antibiotic treatment, the plasma levels of all four metabolites were reduced to undetectable levels (Fig. 3c), demonstrating their dependence on bacterial metabolism in the gut. These four compounds have previously been identified as microbiota-dependent molecules, being either diminished or absent in the gut or plasma of germ-free mice or colectomized humans27–31. It is interesting that IPA was undetectable in the urine of mono-colonized mice and we identified indolepropionylglycine as the secreted form of IPA (Supplementary Fig. 1).
C. sporogenes pathways produce circulating metabolites. It is possible that C. sporogenes colonization might induce pathways in the host to produce the four metabolites that we detected in plasma. To address this possibility, we sought to genetically disrupt the pathways responsible for production of these metabolites. First, we focused on generating a mutant defective in production of 5-AVA. In contrast to Clostridoides difficile and Acetobacterium sticklandii, which have single copy genes in the proline reductase gene cluster, C. sporogenes carries two separate gene clusters encoding multiple copies of proline reductase subunits (Extended Data Fig. 2a). We found that mutants in the proline reductase transcriptional regulator (prdR) slowed the kinetics of 5-AVA production, but this gene was not essential for 5-AVA production (Extended Data Fig. 2b and Supplementary Table 6). By comparison, a mutant in the single copy proline racemase gene (prdF) involved in conversion of L- to D-proline (Fig. 3d) completely abolished 5-AVA production in vitro (Fig. 3e and Supplementary Table 6). Despite producing no 5-AVA, cultures of prdF consumed arginine and accumulated high levels of proline in the supernatant (Extended Data Fig. 2c and Supplementary Table 6), suggesting that arginine metabolism in this mutant is blocked in the conversion of L- to D-proline, the latter being the substrate for proline reductase.

The three reductive products of aromatic amino acid metabolism—PPA, 4-OH-PPA and IPA—are produced by C. sporogenes via a shared pathway (Fig. 3d) involving phenyl-lactate dehydrogenase (fldH), acyl-CoA transferase (fldA), phenyl-lactate dehydrogenase (fldBC) and its activase (fldI), and acyl-CoA dehydrogenase (acdA)30,32. Mutants in the fldC and acdA genes abolished 4-OH-PPA and IPA production, and trace amounts of PPA were produced (Fig. 3e and Supplementary Table 7).

We then colonized germ-free mice with either wild-type (WT) C. sporogenes or its prdF, acdA or fldC mutants, and collected plasma, urine and caecal contents for LC–MS analysis. Despite colonizing to similar levels as the WT (Supplementary Fig. 2), mice colonized by the prdF mutant had undetectable levels of 5-AVA in plasma, urine and caecal contents (Fig. 3f and Supplementary Tables 8–10). Mice colonized by the fldC mutant displayed undetectable plasma levels of IPA and reduced levels of PPA and 4-OH-PPA compared with WT-colonized mice (Fig. 3f). The levels of these three metabolites were also diminished in the caecal contents, demonstrating a concordance between aromatic amino acid metabolism in the gut and levels in the circulation. In contrast, although the acdA mutant was defective in aromatic amino acid metabolism in vitro, the levels of both PPA and 4-OH-PPA in acdA-colonized mice were comparable to WT-colonized mice (Fig. 3f). PPA and 4-OH-PPA in caecal contents of acdA-colonized mice were also similar to those of WT-colonized mice, suggesting that, in vivo, another C. sporogenes gene may compensate for the defect in acdA (possibly the acyl-CoA dehydrogenase, CLOSPO_02759, involved in leucine metabolism). Nevertheless, these data indicate that 5-AVA, PPA, 4-OH-PPA and IPA in the host arise from metabolic pathways in bacteria colonizing the gut.

Stickland metabolites circulate in human blood. We next asked whether these four circulating metabolites are present in human blood. To test this, we obtained 99 plasma samples from healthy human blood donors and quantified metabolites using LC–MS (Supplementary Table 11). Of the four metabolites, IPA was the most prevalent, being detected in the blood of 99% of individuals (Fig. 3g). Consistent with our analysis of gnotobiotic mice (Fig. 3c), IPA concentrations were also the highest, ranging in human plasma from 230 nM to 113 μM (Fig. 3g). PPA and 5-AVA were also detected in most healthy blood donors, whereas 4-OH-PPA levels were lower and present in fewer than half the individuals (Fig. 3g). Our findings in human blood samples are consistent with the model generated from our mouse studies, suggesting that amino acid metabolism by gut bacteria might also influence circulating metabolites in humans. Of these metabolites, IPA is the most concentrated in both gnotobiotic mice and humans, with rare individuals having high concentrations of circulating IPA.

Circulating molecules are reductive Stickland metabolites. C. sporogenes metabolizes amino acids in pairs, coupling the oxidation of one with the reduction of another via the Stickland reaction. Oxidative pathways are thought to yield ATP via substrate-level phosphorylation, and reductive pathways provide redox balance within the cell (Fig. 4a)33,34. In this context, previous studies have suggested that 5-AVA, PPA, 4-OH-PPA and IPA are end-products of reductive Stickland metabolism of proline, phenylalanine, tyrosine and tryptophan, respectively35,36,37.

To test whether these four circulating metabolites arise from metabolism of their cognate amino acids, we performed stable isotope tracing experiments. After growth in defined medium where proline, phenylalanine, tyrosine or tryptophan was individually substituted by its deuterium isotope-labelled proline, phenylalanine and tyrosine were completely consumed by 24 h, being converted to labelled derivatives (5-AVA (d6,d5), PPA (d7,d6,d5) and 4-OH-PPA (d4)) with yields ranging from 65% to 109% (Fig. 4b and Supplementary Table 12). Tryptophan metabolism was comparably slower, but ~93% of tryptophan was consumed by 24 h, being converted to IPA-d5 with a yield of 60% (Fig. 4b). Despite pathways existing in C. sporogenes for the oxidative metabolism of phenylalanine, tyrosine and tryptophan35,36,5, under these experimental conditions only reductive products of these amino acids were detected (Fig. 4c). These findings indicate that C. sporogenes cells convert most of these amino acids to reduced products, rather than using them solely for biosynthetic purposes such as protein synthesis. In cultures where labelled phenylalanine, tyrosine and tryptophan were provided, no unlabelled PPA, 4-OH-PPA or IPA was detected (Extended Data Fig. 3a–c and Supplementary Table 13), suggesting that these metabolites arise from catabolism of their cognate amino acids, not through de novo biosynthetic pathways from other precursors. 5-AVA was an exception, however, with its unlabelled product accumulating in cultures where labelled proline was added (Extended Data Fig. 3d), which we found reflected the capacity of cells to convert unlabelled arginine in the medium to proline and 5-AVA (Extended Data Fig. 3e and Supplementary Table 14).

Next, we tested whether production of the reductive metabolites, 5-AVA, PPA and IPA, is stimulated by provision of an oxidative amino acid partner. Whereas cells incubated with proline or valine alone showed limited substrate consumption and product accumulation (Fig. 4d and Supplementary Table 15), cells incubated with both proline and valine consumed most of the proline and all the valine by 3 h, converting proline to 5-AVA and valine to isobutyrate (Fig. 4d). Similar results were seen with the Phe/Val combination (Fig. 4d); however, the Trp/Val combination yielded a more subtle pattern, where IPA production was low (Fig. 4d), but was significantly higher when valine was added (Extended Data Fig. 4 and Supplementary Table 16). Collectively, these findings demonstrate that 5-AVA, PPA, 4-OH-PPA and IPA are reductive pathway products of coupled Stickland reactions in C. sporogenes.

Reductive Stickland metabolism is coupled to ATP formation. During Stickland metabolism of amino acids, oxidative pathways are thought to yield ATP via substrate-level phosphorylation and reductive pathways provide redox balance within the cell33,34. However, anaerobic bacteria can also use reductive metabolism to fuel anaerobic respiration, providing an additional mechanism to obtain energy. We reasoned that reductive pathways for amino acid metabolism via the Stickland reaction might also serve a role in energy capture for bacteria within the gut. Therefore, we analysed...
Fig. 4 | Circulating metabolites are formed from reductive pathways for Stickland metabolism. a, Overview of the Stickland reaction. Amino acids are metabolized as pairs, with one being oxidized, whereas the other is reduced. The oxidative pathway forms ATP via substrate-level phosphorylation and the reductive pathway serves redox balance. b, Stable isotope tracing. *C. sporogenes* was cultured in a synthetic medium containing 20 amino acids where phenylalanine, tyrosine, tryptophan or proline was individually substituted by their deuterium isotopeologue. Cell-free supernatants were collected at time t = 0, 6 and 24 h and metabolites were detected by LC–MS. Percentages indicate the percentage of substrate converted to product after 24 h. c, End-products of proline, phenylalanine, tyrosine and tryptophan as reductive (red.) Stickland metabolites. ox., oxidative. d, Stable isotope tracing of *C. sporogenes* cell suspensions incubated with oxidative (valine) and reductive (proline, phenylalanine, tryptophan) amino acid pairs. IAA, indoleacetic acid. isoBA, isobutyrate. In b and d, incomplete isotopic purity of substrates and reversible cellular reactions resulting in solvent deuterium exchange led to multiple isotopes, which were summed in the plots. For b and d, data are plotted as means ± s.d. from n = 3 experiments.

the metabolic gene clusters encoding reductive amino acid pathways with an eye to identifying potential mechanisms for energy capture. Inspection of the gene clusters encoding reductive amino acid pathways revealed that each pathway converges on a step involving a multi-subunit electron transfer protein (Fig. 5a). The aryl amino acid reductive gene cluster encodes an acyl-CoA dehydrogenase with two electron transfer factors (EtfAB) sharing 40–60% amino acid identity with the EtfAB–butyryl-CoA dehydrogenase (Etf–Bcd) complex from *Clostridium kluyverii* involved in ethanol fermentation36 (Fig. 5b). Etf–Bcd catalyses flavin-based electron bifurcation, where the energetically downhill reduction of crotonyl-CoA by NADH drives the energetically uphill reduction of ferredoxin by NADH13,37. In so doing, the cell has low-potential reduced ferredoxin at its disposal to drive ion translocation through the Rnf complex with NAD+ as an electron acceptor38. The gene cluster for reductive leucine metabolism, present in *C. sporogenes*39, also encodes a homologue of the Etf–Bcd complex (Fig. 5b). The two proline reductive gene clusters encode a different electron transfer protein, proline reductase (Fig. 5c). Notably, a link to energy conservation for proline reductase has not been established. These metabolic gene clusters suggest that reductive Stickland pathways are linked to electron transfer reactions that might enable ATP synthesis in the cell.

To test this experimentally, we established a whole-cell-based ATP assay using resting cell suspensions of *C. sporogenes*. We validated our assay by analysing ATP formation from trans-cinnamate, a plant-derived metabolite reduced to PPA by the iron–sulfur flavoenzyme, cinnamate reductase (Extended Data Fig. 5a). When *C. sporogenes* was incubated with trans-cinnamate, we observed a rapid rise in cellular ATP levels, followed by a short plateau and a rapid decrease back to resting levels (Extended Data Fig. 5b). This result, which replicates that from previous studies41, provides evidence that cinnamate reductase is involved in energy conservation and validates our approach to measuring ATP levels in *C. sporogenes*.

As phenylalanine, tyrosine, tryptophan and leucine may also be metabolized down oxidative pathways, we supplied cells with pathway intermediates (for example, phenyl-lactate, 3-(4-hydroxyphenyl)lactate, indolelactate, 2-hydroxyisocaproate) specific to reductive pathways. When proline or reductive pathway intermediates for phenylalanine, tyrosine, tryptophan or leucine were added to cells, ATP levels rose rapidly, plateaued and then returned to resting levels (Fig. 5d). As reductive aryl amino acid metabolism (phenylalanine, tyrosine, tryptophan) operates through a shared pathway, we chose to explore ATP formation further using phenylalanine metabolism as a representative of this pathway. These analyses revealed that D-phenyl-lactate (and not its L-stereoisomer) supported ATP formation (Extended Data Fig. 6b and Supplementary Table 17). Cell suspensions incubated with the end-products (Extended Data Fig. 5c) of reductive or oxidative pathways failed to elicit ATP formation (Extended Data Fig. 6d,e). Genetic disruption of the acyl-CoA dehydrogenase gene (acdA) abolished ATP formation from Dl-phenyl-lactate, indicating that acdA is critical for ATP formation during reductive phenylalanine metabolism (Extended Data Fig. 6f). Similar results were seen for metabolism of the tyrosine and tryptophan metabolic intermediates (Dl-4-hydroxyphenyl-lactate and Dl-indolelactate), with the acdA mutant being deficient in ATP formation from these substrates (Extended Data Fig. 66,g,h). Pre-treatment of cells with the protonophore, 3,3’,4,4’,5-tetrachlorosalicylanilide uncoupled Dl-phenyl-lactate and proline metabolism from ATP formation (Extended Data Fig. 7 and Supplementary Table 18), indicating that a proton gradient is involved in ATP formation. These findings reveal that pathways for reductive Stickland metabolism are
Fig. 5 | Reductive metabolism is coupled to ATP formation. a, Pathways for reductive Stickland metabolism that catalyse net 2 electron reductions, each converging on an electron transfer protein. Pyruvate to butyrate is shown as a well-characterized example. αKG, α-Ketoglutarate. b, Gene clusters for reductive metabolism of phenylalanine, tyrosine, tryptophan and leucine share homologues of the electron-bifurcating Bcd-complex, which produces reduced ferredoxin (Fdred). Fdox, oxidized ferredoxin. c, The proline metabolism gene cluster encoding components of the proline reductase enzymes that are implicated in proline-dependent extracellular proton transport. hyp., hypothetical protein. The complete clusters from C. sporogenes are shown in Extended Data Fig. 2a. d, Reductive metabolism of phenylalanine, tyrosine, tryptophan, leucine and proline coupled to ATP formation in C. sporogenes resting cell suspensions. For phenylalanine, tyrosine and tryptophan, cells were incubated with known intermediates in the reductive pathways (for example, phenyl-lactate, 3-(4-hydroxyphenyl)lactate, and indolelactate). For d, experiments were repeated independently three times and representative data are shown.

linked to ATP formation and involve the generation of a proton motive force.

The Rnf complex is important for Stickland metabolism. Having demonstrated that reductive Stickland metabolism is coupled to ATP formation, we next asked whether the Rnf complex might be an important coupling site. We identified a gene cluster that encodes the Rnf complex in C. sporogenes (Fig. 6a) and targeted genes (rnfB and rnfE) encoding two separate subunits for disruption. RnfB is thought to be the entry point for the Rnf complex, accepting electrons from ferredoxin39, and RnfE is thought to be a transmembrane protein involved in ion transport40 (Fig. 6b). These mutants suffered a growth defect when grown in minimal medium containing ten amino acids, yet this phenotype could be suppressed by the addition of glucose to the culture medium (Fig. 6c). These data suggest that the growth phenotype in rnfB and rnfE mutants is characterized by defective amino acid metabolism. To provide more insight into the metabolic defect when the Rnf complex is disrupted, we cultured the WT and rnfB mutant in defined medium +20 amino acids and comprehensively profiled amino acids and Stickland metabolites by LC–MS during growth (Supplementary Table 19). Compared with WT C. sporogenes, the rnfB mutant consumed phenylalanine and tyrosine more slowly, producing more oxidized products from these substrates (Fig. 6d). The rnfB mutant also accumulated reductive pathway intermediates (phenyl-lactate and 3-(4-hydroxyphenyl)lactate), indicating a partial block in reductive metabolism (Fig. 6d). The rnfB mutant produced less IPA from tryptophan and accumulated higher levels of the reductive pathway intermediate, indolelactic acid (Fig. 6d). Consumption of arginine and proline by the rnfB mutant was similar to the WT,
but 5-AVA levels were significantly lower in rnfB supernatants at the 6-h ($P<0.0001$) and 24-h ($P=0.045$) timepoints, eventually reaching WT levels by 48 h (Fig. 6d). It is interesting that the rnfB mutant consumed branched-chain amino acids (valine, isoleucine and leucine) more rapidly than the WT, converting them to oxidative products (isobutyrate, 2-methylbutyrate and isovalerate) (Fig. 6d). These results suggest that, during growth in a defined medium, the rnfB mutant displays a defect in reductive Stickland metabolism, favouring production of metabolites from oxidative pathways. However, reductive Stickland metabolism is not completely blocked, suggesting that the cell uses alternative pathways to dispose of electrons carried by ferredoxin.

Next, we asked whether Stickland metabolites are altered in the Rnf complex mutant in vivo. To test this, we colonized gnotobiotic mice with either WT C. sporogenes or its rnfB mutant and detected metabolites in caecal contents and plasma by LC–MS. By
comparison with WT-colonized mice, rnfB-colonized mice had similar levels of reductive pathway products except for IPA, which was significantly lower in the caecal contents (P = 0.0073) and the plasma (P = 0.0021) of rnfB-colonized mice (Fig. 6e). Isovalerate was significantly lower (P = 0.044) in the caecal contents of rnfB-colonized mice and 4-hydroxyphenylacetylelglycine was lower (P = 0.0058) in the plasma. Other metabolites that were different in the defined medium (Fig. 6d) were not significantly different in the caecal contents or plasma of mono-colonized mice (Supplementary Tables 20 and 21). Thus, although the Rnf complex clearly impacts Stickland metabolism in defined medium, the conditions of the mammalian intestine enable compensation for this metabolic defect.

The Rnf complex is a fitness determinant within the gut. Given our finding that the Rnf complex mutants suffer a growth defect in vitro, we reasoned that the Rnf complex might also be important in vivo. To address this, we performed an in vivo competition experiment comparing the WT and rnfB mutant in germ-free mice. The rnfB mutant was rapidly outcompeted by the WT strain, becoming nearly undetectable in faeces by 7 d post-colonization (Fig. 6f). This finding was recapitulated in the presence of a more complex stably colonized microbial community, revealing the Rnf complex as an important fitness determinant for C. sporogenes during colonization and persistence in the gastrointestinal tract (Fig. 6g and Supplementary Fig. 3). Our results suggest that the Rnf complex enables more efficient metabolism, probably by linking reductive Stickland metabolism to generation of a proton gradient that fuels ATP formation or additional physiological processes such as membrane transport and chemotaxis.

Electron transfer proteins are widely distributed. To assess the prevalence of the genes responsible for production of metabolites described in this study, we searched for homologues in the National Center for Biotechnology Information (NCBI) GenBank database (Supplementary Table 22a–e) and used MetaQuery to search for these proteins in human faecal metagenomic datasets (Supplementary Fig. 4). These analyses revealed the following observations: (1) the Rnf complex is widespread among gut bacterial genomes (Extended Data Fig. 8) and in gut metagenomes; (2) genes for reductive amino acid metabolism are more narrowly distributed than the Rnf complex, showing the following trend in abundance: propionate > aromatic amino acid > leucine metabolism; and (3) genes for proline metabolism are present in most gut metagenomes, but show a wide distribution of abundance, suggesting that there may be large interindividual differences in proline metabolism.

Discussion

Our study demonstrates that coupled metabolism of amino acids via the Stickland reaction contributes to metabolites that accumulate in the mammalian gut and circulate in the blood. These circulating metabolites are known to influence host physiology, with 5-AVA having potential roles in modulation of host behaviour30, 4-OH-PPA modulating host type I interferon signalling42 and IPA activating the pregnane X receptor and influencing intestinal permeability35,43.

Our results show that reductive Stickland metabolism is coupled to ATP formation. For reductive metabolism of leucine, phenylala- nine, tyrosine and tryptophan, the link to ATP formation probably occurs at the level of enoyl-CoA reduction (Fig. 5a). Intriguingly, enoates (or their enoyl-CoA derivatives) are intermediates common to other reductive pathways such as those that produce succinate via fumarate reductase, butyrate via butyryl-CoA dehydrogenase, imidazole propionate via urocanate reductase and hydrocaffeate via caffeoyl-CoA reductase (Extended Data Fig. 9). All these steps are thought to be linked to energy conservation, therefore the favourable redox potential of the enoate is probably used by gut bacteria to fuel anaerobic respiration and boost energy yields.

Our findings suggest that, in C. sporogenes, the Rnf complex enables higher ATP yields, probably by coupling reductive amino acid metabolism to generation of a proton motive force. By re-evaluating ATP economy in the cell (considerations in Supplementary Table 23), reductive pathways may account for ~40% of ATP from Stickland metabolism. The redox-coupled oxidative and reductive Stickland reactions are a key way for proteolytic gut bacteria to produce ATP (Extended Data Fig. 10), and through this metabolism they produce metabolites that accumulate in the gut and circulate within the human body.

Methods

Statement on research compliance and ethical regulation. All experiments in the present study comply with ethical regulations. Animal experiments were performed following a protocol approved by the Stanford University Administrative Panel on Laboratory Animal Care. Strain culture experiments were performed following a protocol approved by the Stanford University Administrative Panel on Biosafety. Metabolonomics studies on human plasma samples were performed with de-identified human blood samples purchased from the Stanford Blood Center.

Reagents used in the present study. All chemicals and reagents used in the present study were of the highest possible purity and are listed in Supplementary Table 24.

Bacterial strains and culture conditions. C. sporogenes ATCC 15579 was obtained from the ATCC. It was routinely cultured in reinforced clostridial medium (RCM) at 37 °C in a CO2 type B anaerobic chamber using gas mix consisting of 5% hydrogen, 10% carbon dioxide and 85% nitrogen, and hydrogen was maintained at ~3.3% using an anaerobic gas insufser. All media and plastic ware were pre-reduced in the anaerobic chamber for at least 24h before use. For growth measurements in defined medium, we used a previously described defined minimal medium referred to as standard amino acid complete medium (SACC) with either 10 or 20 amino acids (1 mM each) and with or without glucose (1 mM) depending on the experiment. C. sporogenes WT or mutants were inoculated into this medium from an overnight culture at a starting OD (600 nm) of ~0.01. Where appropriate, OD measurements were performed in Balch-type anaerobic tubes using a GENESYS 30 spectrophotometer from Thermo Fisher Scientific. For assays in cell suspensions, C. sporogenes was cultured in defined medium with 20 amino acids (SACC) for ~16 h, then subcultured in SACC-20 and grown for ~5 h (OD ~ 0.15). Then cells were harvested by centrifugation and resuspended to an OD of ~1.0 before the addition of substrates.

The Escherichia coli HB101/pRK24 or S17 conjugation hosts were routinely cultured at 30 °C in lysogeny broth (LB), supplemented with tetracycline (12 μg ml−1) to ensure maintenance of the pRK24 plasmid. E. coli TG1 was used for routine cloning. Chloramphenicol (25 μg ml−1) was used for selection of pMTL007C-E2 plasmids in E. coli. Organisms used in the defined community colonization included: Clostridium sporogenes ATCC 15579, Clostridium scindens ATCC 35704, Eubacterium rectale ATCC 33656, Bifidobacterium breve UCC2003, Edwardsiella tarda ATCC 23665, A. butyricum, B. licheniformis VPI-5482 and Bacteroides vulgatus ATCC 8482.

Plasmid construction and cloning. For specific gene disruptions, we used the Intron targeting and design tool on the ClosTron website (http://www.clostron.com/clostron2.php) using the Perutz algorithm. The plasmid contained within the pMTL007C-E2 plasmid was retargeted to the specific sites listed in Supplementary Table 25 and the plasmid vectors were synthesized by DNA 2.0.

Mutant generation using ClosTron. Intron-retargeted Clostron plasmid DNA was introduced into C. sporogenes by conjugation. Plasmids were introduced into E. coli S17 by electroporation and transformants were selected on LB agar plates supplemented with chloramphenicol (20 μg ml−1). E. coli HB101/pRK24 or S17 containing Clostron plasmids were cultured overnight in LB supplemented with chloramphenicol (20 μg ml−1). Then cells (1 ml) were washed twice with equal volumes of phosphate-buffered saline and the pellet was resuspended in 200 μl of an overnight culture of C. sporogenes grown in RCM without agar. Colonies were then spread on RCM agar plates supplemented with chloramphenicol (20 μg ml−1). Then cells (1 ml) were washed twice with equal volumes of phosphate-buffered saline and the pellet was resuspended in 200 μl of an overnight culture of C. sporogenes grown in RCM without agar. Colonies were then spread on RCM agar plates supplemented with chloramphenicol (20 μg ml−1) and thiamphenicol (15 μg ml−1), re-streaked for purity, and individual well-isolated colonies were inoculated into RCM broth (without agar) containing the same antibiotics. After overnight culture, the cells were diluted 10-fold, then 100 μl was spread on an RCM agar plate containing erythromycin (5 μg ml−1). Colonies appearing within ~24 h were picked, re-streaked on RCM agar plates supplemented with erythromycin and well-isolated colonies were inoculated into RCM broth (without agar) supplemented with erythromycin. Genomic DNA (gDNA) was isolated from candidate clones using the DNeasy Blood and Tissue...
Kit from QIAGEN with a lysozyme pre-treatment step, and this DNA was used as a template for PCR using gene-specific primers. Primer sets (Supplementary Table 25) were designed to produce a ~600-bp product for the WT and ~2,800-bp product for the mutant containing the intron. Verified mutants were streaked on to M63 medium for the mutant containing the intron. Reaction mixture was quenched with 0.02% formic acid in 10% acetonitrile:water before LC–MS.

3-Nitrophenylhydrazine derivatization protocol. This derivatization method targets compounds containing a free carboxylic acid. Extracted samples were mixed with 3-nitrophenylhydrazine (NPH; 200 mM in 80% acetonitrile) and N-(3-dime thylaminopropyl)-N’-ethy carbodiimide (120 mM in 6% pyridine) at a 2:1.1 ratio. The plate was sealed with a plastic sealing mat (Thermo Fisher Scientific, catalogue no. AB-6566) and incubated at 40 °C, 600 r.p.m. in a thermomixer for 30–60 min to derivatize the carboxylate-containing compounds. The reaction mixture was quenched with 0.02% formic acid in 10% acetonitrile:water before LC–MS.

Quantification of metabolites by LC–MS. During the course of the present study, several different LC–MS conditions were used (C18 positive derivatized, C18 negative, AB-6566) and incubated at 40 °C, 600 r.p.m. in a thermomixer for 30–60 min to derivatize the carboxylate-containing compounds.

ATP measurements in cell suspensions. WT C. sporogenes cells were streaked from an aerobic glycerol stock on to a TYG (3% w/v tryptone, 2% w/v yeast extract, 0.1% w/v sodium thiosulphate) agar plate and incubated at 37 °C in an anaerobic chamber from Coy Laboratories using gas mix consisting of 5% hydrogen, 10% carbon dioxide and 85% nitrogen, and hydrogen was maintained at ~3.3% using an anaerobic gas infuser. After ~24 h, a well-isolated colony was inoculated into 5 ml of TYG broth and cultured to stationary phase (~24 h). Cells were aliquoted into 20 ml of TYG broth (1,000-fold dilution) and grown to the late-log phase of growth (~16 h). The cells were then harvested by centrifugation (5,000g, 10 min, 4 °C) and washed twice with 20 ml volumes of pre-reduced phosphate assay buffer, or buffer alone, was dispensed into rows of a separate 96-well microplate. ATP was then determined using the DC Protein Assay Kit II from BioRad with bovine serum albumin as an internal standard (Supplementary Table 27).

Short chain fatty acids by gas chromatography–MS. Cultures performed (5% w/v) were acidified with 6 M HCl (50 μl), then diluted in LC–MS-grade water (150 μl). Organic acids were then extracted with one volume of diethyl ether (250 μl). An aliquot (95 μl) of the organic layer was transferred to a new sealed vial, combined with N-tetradecylmethylsilyl-N-methylfluorcarboxamide (5 μl) and derivatized at room temperature for 40 h. Then 1 μl was injected on to an Agilent 7890A gas chromatograph equipped with an Agilent VF-5HT column (30 m × 0.25 mm × 0.1 μm) coupled to an Agilent 5977B mass spectrometer. The inlet was set to 280 °C and injection occurred in split mode with a ratio of 1:10. Oven conditions were as follows: 50 °C for 2 min, ramped to 70 °C at 1 °C min−1, then to 85 °C at 3 °C min−1 and then to 290 °C at 30 °C min−1. The mass spectrometer transfer line was set to 250 °C and the quadrupole was set at 150 °C. MS data were collected under scan mode from 50 to 300 m/z with a 3-min solvent delay.
to remove incomplete sequences (<500 bp) and gaps, and then were re-aligned and a phylogenetic tree was constructed using the neighbour joining method. Representative gene clusters were exported from MultiGeneBlast and visualized in Adobe Illustrator (v.25.1).

Analysis of electron transfer proteins in GenBank. To identify homologues of the electron transfer proteins analysed in the present study, we performed BLASTp searches of the GenBank database. Query sequences included the butyryl-CoA dehydrogenase (Bcd) from C. kluveyri DSM 555 (GenBank accession no. EU35691.1), the (ary)acetyl-CoA dehydrogenase (AcAa) from C. sporogenes ATCC 15579 (GenBank accession no. EDU39257.1), the isocaproyl-CoA dehydrogenase (AcDb) from C. sporogenes ATCC 15579 (GenBank accession no. EDU35691.1), the proline reductase subunit A (PrdA) from C. sticklandii DSM 519 (GenBank accession no. CBH22353.1) and the Rnf protein from C. sporogenes ATCC 15579 (GenBank accession no. EDU37753.1). Our initial analysis of gene cluster neighbourhoods for these homologues using MultiGeneBlast suggested that a cutoff of 65% amino acid identity was necessary to identify possible isofunctional homologues for Bcd, AcAa, AcDb and PrdA, whereas a less stringent cutoff of 50% amino acid identity was suitable for RnfB. These query sequences were used in BLASTp searches of the GenBank database (30 December 2020) and homologues with <80% coverage over the length of the proteins were discarded.

Analysis of abundance and prevalence of electron transfer proteins in metagenomics datasets. To assess the abundance and prevalence of electron transfer proteins (Bcd, AcAa, AcDb, PrdA and RnfB) in human faecal metagenomic datasets, we employed the web-based tool, MetaQuery®. Query sequences included the Bcd from C. kluveyri DSM 555 (GenBank accession no. EDU35691.1), the AcAa from C. sporogenes ATCC 15579 (GenBank accession no. EDU39257.1), the AcDb from C. sporogenes ATCC 15579 (GenBank accession no. EDU35691.1), the PrdA from C. sticklandii DSM 519 (GenBank accession no. CBH22353.1) and the Rnf protein from C. sporogenes ATCC 15579 (GenBank accession no. EDU37753.1). Owing to limitations in threshold selections implemented in MetaQuery®, we used a slightly lower threshold for amino acid percentage identity of 60% for Bcd, AcAa, AcDb and PrdA to identify homologues in metagenomics datasets.

Statistics and reproducibility. No statistical method was used to predetermine sample size. Sample sizes were chosen based on animal litter numbers and controlling for sex and age within experiments. No data were excluded from the analyses. For LC-MS, samples were randomized before injection and analysis. For all animal experiments, littermates were grouped by a researcher unaware of the experimental design. Grouped littermates were assigned to treatment arms randomly before starting experiments. Researchers colonizing and sampling mice and performing measurement and data analysis of bacterial growth, ATP formation, qPCR and MS assays were not blinded to experimental group due to the impracticality of setting up and performing the assays in a blinded fashion. However, the results of all of these experiments were measured via quantitative metrics, reducing the risk of bias in the results. Data distribution was assumed to be normal but this was not formally tested.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The authors declare that the data supporting the findings of the present study are available within the paper and the Supplementary Information. Genome sequences analysed as part of the NHM Human Microbiome Project are available on NCBI GenBank under BioProject accession no. 43021.

Code availability
No customized code was used in the present study.

Received: 7 January 2022; Accepted: 24 March 2022; Published online: 2 May 2022

References
1. Van Treuren, W. & Dodd, D. Microbial contribution to the human metabolome: implications for health and disease. Annu. Rev. Pathol. 15, 345–369 (2020).
2. Russell, W. R. et al. Major phenylpropanoid-derived metabolites in the human gut can arise from microbial fermentation of protein. Mol. Nutr. Food Res. 57, 523–535 (2013).
3. Smith, E. A. & Macfarlane, G. T. Dissimilatory amino acid metabolism in human colonic bacteria. Anaerobe 3, 327–337 (1997).
4. Liu, Y., Hou, Y., Wang, G., Zheng, X. & Hao, H. Gut microbial metabolites of aromatic amino acids as small molecules in host–mucobio interplay. Trends Endocrinol. Metab. 31, 818–834 (2020).
5. Allison, M. J., Bryant, M. P. & Doetsch, R. N. Volatile fatty acid growth factor for cellulolytic cocci of bovine rumen. Science 128, 474–475 (1958).
Extended Data Fig. 1 | *C. sporogenes* pathways converge on proline reductase. Arginine, citrulline, ornithine, and trans-4-hydroxy-L-proline pathways all converge on L-proline which is converted to D-proline by proline racemase. D-proline is then converted to 5-aminovalerate by the enzyme, proline reductase. Locus tag IDs from the *C. sporogenes* ATCC 15579 genome are provided next to each of the enzyme-catalyzed steps. For the proline reductase enzyme, the locus tag IDs are provided for each of the two gene clusters which contain multiple copies of proline reductase enzyme subunits.
Extended Data Fig. 2 | Proline reductase gene clusters and in vitro phenotypes of Clostridium sporogenes mutants. A) The two gene clusters for proline metabolism in C. sporogenes encode multiple copies of the prdA, prdB, prdC, and prdG genes. Red arrows designate mutants tested in this study for proline metabolism. Locus tag IDs for the two gene clusters are provided. B) Wild-type C. sporogenes and two ClosTron insertional mutants in prdR were cultured in defined medium containing 20 amino acids and 5-aminovalerate in the supernatant was quantified at 3, 6, 24, and 48 h by LC-MS. Numbers after the prdR name indicate positions in the gene where the group II intron was integrated. Experiments were performed in triplicate and data are reported as means ± standard deviations. C) Wild-type C. sporogenes and a prdF mutant were cultured in defined medium containing 20 amino acids and arginine and proline in the supernatant was quantified at 3, 6, 24, and 48 h by LC-MS. Data are plotted as means ± standard deviations from n = 3 experiments.
Extended Data Fig. 3 | Stickland metabolites do not arise from de novo biosynthetic pathways. A-D) Stable isotope tracing. *C. sporogenes* was cultured in a synthetic medium containing 20 amino acids where Phe, Tyr, Trp, and Pro were individually substituted by their deuterium isotopologues. Cell-free supernatants were collected at t = 24 h and metabolites were detected by LC-MS. Data are plotted as means ± standard deviations from n = 3 cultures. A-C) For phenylpropionate, 3-(4-hydroxyphenyl)propionate, and indolepropionate, no unlabeled products were detected when isotopically labeled amino acids were provided. D) Unlabeled 5-aminovalerate levels were reduced when isotopically labeled Pro was supplied, but ~1 mM 5-aminovalerate was still detected suggesting another source exists for its production. E) Stable isotope tracing of *C. sporogenes* cell suspensions incubated with stable isotopically labeled arginine (Arg-d7). When cells were incubated with Arg-d7, labeled proline and 5-aminovalerate were detected, suggesting that arginine is a substrate for 5-aminovalerate production. Data are plotted as means ± standard deviations from n = 3 experiments.
Extended Data Fig. 4 | Indolepropionate (IPA) and phenylpropionate (PPA) production is stimulated by incubation with oxidative Stickland amino acids. A) C. sporogenes cell suspensions were incubated with stable isotopically labeled tryptophan (Trp-d5) alone or in combination with oxidative Stickland amino acids (Ile, Leu, Val, or Met), then labeled IPA-d5 was measured by LC-MS. IPA-d5 levels were stimulated by addition of Leu, Val, or Met. B) C. sporogenes cell suspensions were incubated with stable isotopically labeled phenylalanine (Phe-d8) alone or in combination with oxidative Stickland amino acids (Ile, Leu, Val, or Met), then labeled PPA-d6 was measured by LC-MS. PPA-d6 levels were stimulated by addition of Leu, Val, or Met. Data are plotted as means ± standard deviations from n = 3 experiments.
Extended Data Fig. 5 | Cinnamate reduction is coupled to ATP formation in *C. sporogenes*. A) The cinnamate reductase gene encodes a unique FAD/[FeS]/FMN containing enzyme with separate binding sites for NADH, cinnamate, and the artificial electron carrier methylviologen, however the natural electron carrier remains unknown. B) Resting cell suspensions of *C. sporogenes* accumulate ATP after being incubated with cinnamate. Buffer control is shown. For B, experiments were repeated independently three times and representative data are shown.
Extended Data Fig. 6 | Reductive metabolism of D-phenyllactate is coupled to ATP formation involving acdA. A) D- or L-phenyllactate was added (1 mM) to resting cell suspensions of C. sporogenes, and ATP levels were measured at indicated time points using a luciferase-based assay. B) Reductive (PPA) and oxidative (PAA) pathway end products were measured by LC-MS during D-phenyllactate metabolism. PPA, phenylpropionate; PAA, phenylacetate. C) Pathways for oxidative and reductive metabolism of phenylalanine showing the position of AcdA in the pathway. D-E) Phenylacetate (D) or phenylpropionate (E) was added (1 mM) to resting cell suspensions of C. sporogenes, and ATP levels were measured at indicated time points using a luciferase-based assay. F-H) DL-phenyllactate (F), DL-3-(4-hydroxyphenyl)lactate (G), or DL-indolelactate (H) was added (1 mM) to resting cell suspensions of WT or acdA mutant C. sporogenes, and ATP levels were measured at indicated time points using a luciferase-based assay. For A-B, D-H, experiments were repeated independently three times and representative data are shown.
Extended Data Fig. 7 | The protonophore, 3,3′,4,5-tetrachlorosalicylanilide (TCS), uncouples reductive Stickland metabolism from ATP formation. Resting cell suspensions of C. sporogenes were preincubated with ethanol (vehicle) or varying concentrations of TCS for 30 min. Then substrates (DL-phenyllactate (DL-PLA) or proline (Pro)) were added, and aliquots were taken at different time-points and quenched in DMSO. Total cellular ATP was quantified using a luciferase-based assay and normalized to total cellular protein and substrates/metabolites were quantified using LC-MS.

A) Dose dependent decrease in ATP formation from DL-phenyllactate with increasing concentrations of TCS. B) Conversion of DL-phenyllactate to phenylpropionate (PPA) at the 15 min timepoint remains constant with increasing TCS while (C) ATP production diminishes. D) Conversion of DL-phenyllactate to phenylpropionate (PPA) increases over time irrespective of TCS levels. E) Dose dependent decrease in ATP formation from Pro with increasing concentrations of TCS. F) Conversion of Pro to 5-aminovalerate (5-AVA) at the 15 min timepoint decreases but is not completely blocked with increasing TCS while (G) ATP production diminishes becoming negligible at 100 μM TCS. H) Conversion of Pro to 5-aminovalerate (5-AVA) increases over time, and while the rate of conversion decreases with increasing TCS levels, proline conversion to 5-aminovalerate is complete by the 60 min timepoint.
Extended Data Fig. 8 | The Rnf gene cluster is widely distributed among reference genomes from the Human Microbiome Project. A) 16S rRNA tree of organisms from the human microbiome reference genome collection determined to have Rnf gene clusters as determined by BLASTp and manual inspection of gene neighborhoods using MultiGeneBlast. B) Rnf gene clusters for select organisms (corresponding to triangles in panel A), grouped by phyla.
Extended Data Fig. 9 | The enoate is a chemical moiety common to pathways for microbial metabolites. Enoates or their enoyl-CoA derivatives are common intermediates that serve as alternate electron acceptors for anaerobic respiration. Enzymes catalyzing the reduction of enoates or enoyl-CoAs are shown.
Extended Data Fig. 10 | Revised model for *C. sporogenes* Stickland metabolism and its contribution to gut bacterial derived metabolites in the gut.

Oxidative pathways yield ATP directly via substrate level phosphorylation, whereas reductive pathways generate reduced ferredoxin via flavin-based electron bifurcation contributing to a proton (or sodium ion) motive force via the Rnf complex. Protons or sodium ions translocated by the Rnf complex re-enter the cell via the membrane bound ATP synthase, resulting in the synthesis of ATP. The membrane gradient may also contribute to other physiological processes such as membrane transport and chemotaxis (not shown).
Reporting Summary

Nature Portfolio wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Portfolio policies, see our Editorial Policies and the Editorial Policy Checklist.

Statistics
For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- n/a
- Confirmed
- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
  Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
  Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code
Policy information about availability of computer code

Data collection
- Growth curves were collected manually or with Gen5 (BioTek).
- Mass spectrometry data was collected using MassHunter LC/MS data acquisition software v.10.1 (Agilent).
- ATP assay data were collected with Gen5 (BioTek).
- Q-PCR data was collected with MxPro (Agilent).

Data analysis
- Growth curve data were analyzed using Microsoft Excel and GraphPad Prism.
- Mass spectrometry data was analyzed using Agilent MassHunter Quantitative Analysis software v.10.0, Microsoft Excel, and GraphPad Prism v.9.1.0.
- ATP assays were analyzed with Microsoft Excel.
- Q-PCR data was analyzed with MxPro and Excel.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.
Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy.

The data supporting the findings of this study are available within the paper and the Supplementary Information. Genome sequences analyzed as part of the NIH Human Microbiome Project are available on NCBI GenBank under BioProject ID 43021.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- Life sciences
- Behavioural & social sciences
- Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/hr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size       | No statistical methods were used to predetermine sample sizes. Sample sizes were chosen based on animal litter numbers and controlling for sex and age within experiments. |
|-------------------|------------------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions   | No data were excluded.                                                                                                                                 |
| Replication       | Experiments were replicated independently 3 times and the findings were reproducible. Gnotobiotic experiments were performed once with 5-9 biological replicates and the findings were reproducible. |
| Randomization     | For LC-MS, samples were randomized before injection and analysis. For all animal experiments, littermates were grouped by a researcher unaware of the experimental design. Grouped littermates were assigned to treatment arms randomly prior to beginning experiments. |
| Blinding          | Researchers colonizing and sampling mice and performing measurement and data analysis of bacterial growth, ATP formation, Q-PCR, and mass spectrometry assays were not blinded to experimental group due to impracticality of setting up and performing the assays in blinded fashion. However, results of all of these experiments were measured via quantitative metrics, reducing risk of bias in the results. |

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
|----------------------------------|---------|
| n/a                              | n/a     |
| - □ Antibodies                   | - □ ChiP-seq |
| - □ Eukaryotic cell lines        | - □ Flow cytometry |
| - □ Palaeontology and archaeology| - □ MRI-based neuroimaging |
| - □ Animals and other organisms  |         |
| - □ Human research participants  |         |
| - □ Clinical data                |         |
| - □ Dual use research of concern |         |

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

| Laboratory animals | Swiss Webster germ free mice (sex-matched, 8-12 weeks of age, n = 5-9 per group) originally obtained from Taconic Biosciences (Hudson, NY) were maintained in aseptic isolators. Mice were maintained on standard chow (LabDiet Cat. # 5K57) and sterile water with access to food and water ad libitum in a facility on a 12-hour light/dark cycle with temperature controlled between 20-22°C and humidity between 40-60%. |

|
| Category                | Description                                                                 |
|-------------------------|-----------------------------------------------------------------------------|
| Wild animals            | The study did not involve wild animals.                                     |
| Field-collected samples | The study did not involve samples collected from the field.                 |
| Ethics oversight        | Animal experiments were performed following a protocol approved by the Stanford University Administrative Panel on Laboratory Animal Care. |

Note that full information on the approval of the study protocol must also be provided in the manuscript.