Host succinate is an activation signal for Salmonella virulence during intracellular infection

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Host succinate is a signal for activation of *Salmonella* virulence during intracellular infection

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Abstract

Key to the success of intracellular pathogens is the ability to sense and respond to a changing host cell environment. Macrophages exposed to microbial products undergo metabolic changes that drive inflammatory responses. However, the role of macrophage metabolic reprogramming in bacterial adaptation to the intracellular environment has not been explored. Here, using metabolic profiling and dual RNA-seq, we show that succinate accumulation in macrophages is sensed by intracellular *Salmonella* Typhimurium (*S.*Tm) to promote antimicrobial resistance and type III secretion. *S.*Tm lacking the succinate uptake transporter DcuB display impaired survival in macrophages and in mice. Thus, *S.*Tm co-opts the metabolic reprogramming of infected macrophages as a signal that induces its own virulence and survival, providing an additional perspective on metabolic host–pathogen crosstalk.

One-sentence summary: Host metabolic reprogramming, necessary for immune activation, is co-opted by *Salmonella* to induce virulence during intracellular infection

31 Studies of the interactions between *Salmonella enterica* serovar Typhimurium (*S.*Tm) and
32 macrophages have elucidated both a complex virulence program executed by the pathogen
33 and the opposing ability of macrophages to recognize and kill invading bacteria (*1*). *S.*Tm has
34 evolved to sense the transition from extracellular to intravacuolar environments,
35 characterized by physiological host processes which aim to eliminate the invading pathogen
36 (*2*). These intracellular cues provide signals that activate bacterial two-component systems,
37 such as PhoPQ and PmrAB, which promote resistance to host antimicrobial peptides (*3*), and
38 type III secretion systems, located on the *Salmonella* pathogenicity island 2 (SPI-2), which
39 enables intracellular replication (*4*). Macrophage detection of invading bacteria via pattern
40 recognition receptors results in disruption of the citric acid (TCA) cycle, which shifts host
41 metabolism from oxidative phosphorylation toward aerobic glycolysis with consequent
42 accumulation of several metabolic intermediates (*5*). Although it is increasingly understood
43 that these metabolic changes are key to the regulation of host proinflammatory responses
44 during infection (*6*), the impact of host metabolic reprogramming on intracellular
45 pathogens is largely unknown.
46
47 To determine whether host-derived metabolites modulate the intracellular lifestyle of *S.*Tm,
48 we sought to test their effects on *S.*Tm virulence and survival in macrophages. We utilized 2-
49 deoxyglucose (2DG) to inhibit the shift from oxidative phosphorylation (OXPHOS) to
50 glycolysis (*5*) in activated bone marrow–derived macrophages (BMDMs) infected with *S.*Tm
51 (fig. S1A). We optimized an effective 2DG dose at a low concentration of 1 mM to minimize
52 off-target effects (Fig. 1A and fig. S1, B and C). Additionally, 2DG may lead to a deficient
53 glucose homeostasis of the bacteria that induces a stress response (*7*). 2DG treatment of *S.*Tm
54 in culture media caused an impaired growth and induction of stress response genes, that was
55 abolished in an *S.*Tm mutant lacking the transporter of 2DG, ManZ (*8*) (fig. S1, D to F and
56 table S1). Thus, 2DG and the Δ*manZ* mutant can serve as a tractable model to study the
57 impact of macrophage metabolic reprogramming on intracellular *S.*Tm.
58
59 To assess the interplay of host metabolism and pathogen dynamics in our model, we applied a
60 dual RNA-seq method (*9*). We curated a list of 977 macrophage genes that were induced in
61 infected cells (table S2). Host responses to infection with the WT or Δ*manZ* were similar (fig.
62 S2A) and 2DG did not induce transcriptional changes in uninfected (naïve) cells (fig. S2B).
63 We classified host genes that are upregulated during infection into three classes, based on
64 their expression changes by 2DG (Fig. 1B, fig. S2C and table S2). Importantly, class 2

65 composed of genes downregulated by 2DG, including genes that are differentially expressed
66 by metabolic reprogramming, verified glycolysis inhibition by 2DG (Fig. 1C and fig. S2D).
67
68 To test the impact of inhibiting host OXPHOS-to-glycolysis shift on intracellular *S.*Tm, we
69 curated 960 bacterial genes that were upregulated during infection and differentially
70 expressed with 2DG treatment (fig. S3A and table S3). Expression of stress response genes in
71 WT 2DG-treated cells (fig. S3B), and of *ripA,* part of the itaconic acid degradation operon
72 (*10*) in Δ*manZ* strain (fig. S3C), suggested that the macrophage metabolic state is sensed by
73 intracellular *S.*Tm. Importantly, global regulon analysis (table S4) indicated decreased
74 expression in Δ*manZ*-infected, 2DG-treated cells of regulons that mediate intracellular
75 survival in macrophages, most prominently the SPI-2 regulon (hypergeometric test, $P<0.007$,
76 $Q<0.12$; Fig. 1, D and E and fig. S3, D and E). Thus, there is a clear correlation between the
77 metabolic state of infected macrophages and *S.*Tm virulence gene expression.
78
79 We next analyzed intracellular accumulation of host metabolites during infection with *S.*Tm.
80 Several metabolites, including primary metabolites of the TCA, were significantly changed
81 upon *S.*Tm infection and also reduced by 2DG treatment (Fig. 2A, fig. S4A, and table S5). To
82 test whether *S.*Tm responds to the accumulation of these host metabolites, we performed a
83 virulence assay utilizing the antimicrobial peptide polymyxin B (PMB). Survival of a Δ*phoP*
84 strain, that is sensitive to PMB challenge (*11*), was significantly restored only in the presence
85 of succinate and itaconate (Fig. 2B and fig. S4B). *S.*Tm grew normally in medium
86 supplemented with succinate, but not with itaconate, at concentrations found within activated
87 macrophages (*12*) (fig. S4, C and D). Thus, we hypothesized that succinate serves as a signal
88 to induce *S.*Tm virulence.
89
90 Using ^{14}C-labeled succinate, we found that *S.*Tm took up and accumulated exogenous
91 succinate (Fig. 2C). We selected a subset of SPI-2 candidate genes whose expression
92 correlated with metabolic remodeling of infected macrophages (from Fig. 1D). We measured
93 induced mRNA expression and fluorescence from transcriptional reporters of these genes in
94 succinate-containing media (Fig. 2, D and E and fig. S4E). Succinate also induced protein
95 levels of one of these SPI-2 effectors, SseL (Fig. 2F). Finally, *S.*Tm grown in succinate-
96 containing media showed increased survival in BMDMs compared to control (Fig. 2G). Thus,
97 succinate is sufficient to mount a bacterial pathogenic program.

98

99 Using RNA-seq, we comprehensively analyzed the direct effects of succinate on *S.*Tm (table
100 S6). Succinate induced the expression of several regulons known to mediate *S.*Tm virulence
101 within macrophages (Fig. 3A, fig. S5A, and table S7). In line with the observed effect of host
102 metabolic shift on induction of SPI-2 genes during intracellular infection, growth in
103 succinate-containing media significantly induced expression of the SPI-2 regulon
104 (hypergeometric test, $Q<0.001$). Furthermore, succinate induced the expression of the
105 PmrAB regulon (hypergeometric test, $Q<0.001$), important for antimicrobial peptide
106 resistance (*3*) (Fig. 3B). We tested whether succinate-induced PMB resistance (Fig. 2B) is
107 mediated by PmrA-regulated genes (Fig. 3C, fig. S5B). Similar to the Δ*phoP* strain, the
108 Δ*pmrA* strain was susceptible to PMB in control media. However, succinate exposure did not
109 induce PMB resistance in Δ*pmrA*. Resistance was preserved in a Δ*phoP* PmrA^{c} strain, but not
110 in a Δ*pmrA* PhoP^{C} strain. Thus, succinate-mediated PMB resistance appears to be through
111 induction of PmrA-regulated virulence genes, which deviates from the canonical activation
112 by PhoPQ (*13*). Succinate also induced resistance to the intracellular antimicrobial peptide,
113 defensin (*14*) (Fig. 3D and fig. S5C), suggesting a role for succinate in protection against
114 antimicrobial peptides during intracellular infection within macrophages.
115
116 During gut colonization, *S.*Tm can utilize succinate generated by the gut microbiota to
117 complete the TCA cycle (*15*). To test this possibility, we generated an *S.*Tm double mutant
118 lacking *phoP* and the succinate dehydrogenase complex, subunit A (Δ*sdhA* Δ*phoP*). Similar
119 to Δ*phoP*, Δ*sdhA* Δ*phoP* bacteria grown with succinate showed restored resistance to PMB
120 (Fig. 3E and fig. S5, D and E). This result is consistent with succinate acting as a signal for
121 *S.*Tm virulence independent of its role in central metabolism.
122
123 Shifts in host succinate levels might be sensed in the bacterial outer membrane or accumulate
124 within the bacterial cytoplasm. Preferred sugars, such as glycerol, induce carbon catabolite
125 repression, which prevents succinate uptake (*16*). Bacteria grown with succinate alone
126 exhibited a dose-dependent induction of the SPI-2 gene *steC*, which was lost in media
127 containing glycerol (Fig. 4A and fig. S6, A and B). The addition of permeable succinate
128 (diethylsuccinate) resulted in elevated *steC* expression even in the presence of glycerol, or of
129 media from activated macrophages (fig. S6C). Thus, succinate is actively transported into
130 bacteria in order to induce *S.*Tm pathogenicity.
131

132 Succinate transport into bacteria is mediated by C4-dicarboxylate transporters (*17*). Growth
133 in succinate-containing media increased expression of three C4-dicarboxylate transporters
134 (Fig. 4B). We examined intracellular survival of the three transporter mutants and measured a
135 significantly lower survival of the Δ*dcuB* mutant (Fig. 4C and fig. S6D). Expression of *dcuB*
136 was induced by growth in succinate-containing media and within BMDMs (fig. S6, E and F).
137 Since genetic redundancy may mask individual transporter phenotypes (*18*), we assessed
138 succinate uptake in a triple dcu mutant (Δ3) background. Succinate uptake was inhibited in
139 the Δ3 mutant and was significantly restored by complementation of DcuB (Fig. 4D). Similar
140 effects were measured for the expression of the SPI-2 effector *steC* (fig. S6G). In
141 macrophages infected with a WT or Δ*dcuB* strains, secretion of SseL SPI-2 effector to the
142 host cytosol was significantly reduced in the Δ*dcuB* mutant (Fig. 4E). Thus, active transport
143 of succinate through DcuB is necessary for *S.*Tm virulence within macrophages.
144
145 To test the role of succinate uptake during in vivo infection, mice were intraperitoneally (i.p.)
146 infected either with WT and Δ*dcuB* mutant or with a Δ*phoP* and a Δ*phoP* Δ*dcuB* mutants
147 (Fig. 4F). There was no significant difference in the competitive index between the WT and
148 Δ*dcuB*. By contrast, the Δ*phoP* Δ*dcuB* had significantly lower survival compared to the
149 Δ*phoP* mutant at 48 and 96 hours after infection (Paired *t* test $P<0.012$, $P<0.0003$
150 respectively). Thus, the relationship between host accumulation of succinate and bacterial
151 virulence, mediated by DcuB, is also relevant for in vivo infection.
152
153 This work indicates that *S.*Tm has co-opted the metabolic reprogramming of infected
154 macrophages as a signal that induces its own virulence (fig. S7). Other studies reinforce the
155 importance of host and microbiota metabolic products that promote colonization by *S.*Tm and
156 other pathogens (*19–21*). Our study indicates that during intracellular infection, uptake of
157 host succinate by the DcuB transporter converges on a unique pathway for virulence
158 induction. One plausible mechanism could be by direct interaction of metabolites with
159 bacterial proteins, as with host proteins (*5*). We suggest a model whereby bacterial virulence
160 is regulated by sensing of TCA intermediates following macrophage metabolic shift, that may
161 be a general theme required for the pathogenic success of diverse intracellular bacteria.
162
163

164 **References and Notes**

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Author contributions

G.R. and R.A. designed the study. G.R., D.H., C.C.M., H.B.A., L.V., S.H.A., and S.B. performed the experiments. M.F. and E.O. performed ^{14}C succinate uptake experiments. M.I. and S.M. performed metabolomics experiments. D.Y. and N.N. analyzed the data. G.R. and R.A. wrote the manuscript.

Competing Interests

The authors declare no competing interests

Data and materials availability

All RNA-seq data have been deposited in NCBI's Gene Expression Omnibus (GEO) under the super-series accession number GSE139208. All data are available in the manuscript or the supplementary materials.

Supplementary Materials

Material and Methods

Figures S1-S7

Tables S1-S10

Supplementary references (*22-40*)

334 **Figure legends**

335

336 **Figure 1: Interplay between macrophage metabolic reprogramming and *S.*Tm**
337 **virulence.**
338 **(A)** BMDMs were infected with *S.*Tm and treated with 2DG; basal respiration (OCR, gray)
339 and stimulated glycolysis (ECAR, black) were measured. Error bars indicate means ± SEM of
340 3-6 replicates representative of three independent experiments. **(B)** Heatmap of host genes
341 upregulated in *S.*Tm-infected BMDMs compared to uninfected controls. Genes were
342 classified into three classes based on their expression changes with 2DG. Colorbars: relative
343 gene expression (red–blue) and fold change between ±2DG (yellow–purple). **(C and D)** Host
344 class 2 genes and bacterial SPI-2 genes presented as the calculated difference in expression
345 between 2DG-treated and untreated samples. **(E)** MA plots of bacterial transcripts in 2DG-
346 treated over untreated samples (*y*-axis) versus the average absolute read counts (*x*-axis).
347

348 **Figure 2: *S.*Tm virulence is induced in response to succinate.**

349 **(A)** BMDMs were infected with a Δ*manZ* strain, treated with 2DG, and intracellular levels of
350 central metabolites were measured by mass spectrometry. **(B)** Δ*phoP* mutant grown without
351 (control; gray) or with PMB (blue) supplemented with 10 mM of the indicated metabolites.
352 Survival was measured by CFU. (**C**) *S.*Tm was cultured in the indicated media supplemented
353 with ^{14}C labeled succinate, and succinate uptake was measured. Background indicates heat-
354 killed bacteria. **(D-G)** *S.*Tm was grown in control media or media supplemented with 10 mM
355 succinate. The expression of *steC, sseL*, and *gapA* (normalizing control) was measured by
356 quantitative real-time PCR **(D)**. Fluorescence signal was measured from *S.*Tm strains
357 expressing a transcriptional reporter fused to selected SPI-2 promoters (normalized to OD_{600})
358 **(E)**. Immunoblot of SseL protein (SseL-2HA) and GroEL **(F)**. BMDMs were infected with
359 *S.*Tm and intracellular survival was measured by CFU (**G**).
360 Error bars indicate means ± SEM, representative of two [(C)] or three [(B), (D-G)]
361 independent experiments. Unpaired *t* test: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.
362

363 **Figure 3: Succinate induces SPI-2 and antimicrobial resistance.**

364 **(A)** RNA-seq analysis of *S.*Tm grown in control media or succinate-containing media. SPI-2
365 and PmrAB regulons were significantly induced in succinate. **(B)** mRNA expression of genes
366 induced by succinate (taken from A) across the PMB resistance locus. **(C to E)** *S.*Tm strains
367 were grown in the presence of PMB **(C and E)** or defensin **(D),** and survival measured by
368 CFU. Error bars indicate means ± SEM, representative of three independent experiments.
369 Unpaired *t* test: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.
370

371 **Figure 4: Uptake of host succinate is required for intracellular survival of *S.*Tm.**

372 **(A)** *S.*Tm harboring a PsteC-*cfp* reporter was grown in media without or with glycerol, and
373 succinate or diethylsuccinate as indicated, and fluorescence was measured (normalized to
374 OD_{600}). **(B)** Expression of the C4-dicaboxylate transporter genes taken from RNA-seq data of
375 Fig. 3A. **(C)** BMDMs were infected with the indicated *S.*Tm strains and intracellular survival
376 was measured by CFU. (**D**) *S.*Tm strains were cultured in succinate-containing media, and
377 succinate uptake was measured by ^{14}C labeled succinate. (**E**) Macrophages were infected
378 with WT or Δ*dcuB* strains. SseL protein was quantified by protein immunoblot of bacterial
379 pellet and host cell cytosol fractions. Boxplots: SseL protein abundance. **(F)** Groups of mice
380 (boxes) were infected i.p. with either WT and Δ*dcuB* or Δ*phoP* and Δ*phoP* Δ*dcuB* mutants,
381 and competitive index was measured by CFU.

382 Error bars indicate means ± SEM, representative of two [(B), (D), (F)] or three [(A), (C), (E)]
383 independent experiments. Unpaired *t* test: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.
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