Stress-Induced Block in Dicarboxylate Uptake and Utilization in *Salmonella enterica* Serovar Typhimurium

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ABSTRACT Bacteria have evolved to sense and respond to their environment by altering gene expression and metabolism to promote growth and survival. In this work, we demonstrate that *Salmonella* displays an extensive (>30-h) lag in growth when subcultured into medium where dicarboxylates such as succinate are the sole carbon source. This growth lag is regulated in part by RpoS, the RssB antiadaptor IraP, translation elongation factor P, and to a lesser degree the stringent response. We also show that small amounts of proline or citrate can trigger early growth in succinate medium and that, at least for proline, this effect requires the multifunctional enzyme/regulator PutA. We demonstrate that activation of RpoS results in the repression of *dctA*, encoding the primary dicarboxylate importer, and that constitutive expression of *dctA* induces growth. This dicarboxylate growth lag phenotype is far more severe across multiple *Salmonella* isolates than it is in its close relative *Escherichia coli*. Replacing 200 nucleotides of the *Salmonella* *dctA* promoter region with that of *E. coli* was sufficient to eliminate the observed lag in growth. We hypothesized that this *cis*-regulatory divergence might be an adaptation to *Salmonella*'s virulent lifestyle, where levels of phagocyte-produced succinate increase in response to bacterial LPS; however, we found that impairing *dctA* repression had no effect on *Salmonella*'s survival in acidified succinate or in macrophages.

IMPORTANCE Bacteria have evolved to sense and respond to their environment to maximize their chance of survival. By studying differences in the responses of pathogenic bacteria and closely related nonpathogens, we can gain insight into what environments they encounter inside of an infected host. Here, we demonstrate that *Salmonella* diverges from its close relative *E. coli* in its response to dicarboxylates such as the metabolite succinate. We show that this is regulated by stress response proteins and ultimately can be attributed to *Salmonella* repressing its import of dicarboxylates. Understanding this phenomenon may reveal a novel aspect of the *Salmonella* virulence cycle, and our characterization of its regulation yields a number of mutant strains that can be used to further study it.

KEYWORDS DctA, *Escherichia coli*, IraP, RpoS, *Salmonella*, dicarboxylates, gene regulation, metabolism, succinate, virulence regulation

Bacteria must adapt to changing environmental conditions by sensing their surroundings and integrating signals to initiate rapid growth in nutrient-rich situations or instigate defense mechanisms and metabolic hibernation in response to stress (1). Pathogens like *Salmonella* have adapted to survive and replicate within their particular host niche, which is reflected in a variety of subtle differences in regulation compared to their nonpathogenic relatives. Understanding the differences in the related pathogenic and commensal bacteria *Escherichia coli* and *Salmonella* has yielded a wealth of insight regarding the challenges pathogens face in the host environment. These bacteria are closely related, yet *Salmonella* has acquired a number of adaptations that accommodate its virulent lifestyle, for example, those allowing *Salmonella* to

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invade tissues and survive within host cells such as macrophage, which is important for *Salmonella* virulence (2–6).

Metabolic modulation is an important component in the adaptation to multiple types of stress. In response to amino acid starvation or other cellular stress cues, RelA or SpoT produces the second messenger molecule, guanosine 5'-disphosphate 3'-diphosphate (ppGpp), to instigate the bacterial stringent response (7–10). Bacteria can also alter gene expression by using the general stress response sigma factor RpoS (σ^S), which has been linked to virulence in a number of pathogenic bacteria by contributing to virulence gene expression and survival within an infected host (11–13). RpoS can be activated in response to a variety of conditions, including starvation, hyperosmolarity, and oxidative stress, and can be regulated at all levels of synthesis from transcription to protein degradation, where it is recognized by the adaptor RssB (also known as MviA, SprE, or ExpM) and chaperoned to the ClpXP protease (14–18). In response to specific stresses, the antiadaptors IraP, RssC (IraM in *E. coli*), and IraD, which bind to and impair RssB to thereby rapidly stabilize RpoS, can be induced (19–21). Strains with reduced *rpoS* activity have been demonstrated to grow faster than wild-type *E. coli* when grown using the dicarboxylate succinate as a sole carbon source (22–24). Furthermore, aerobic growth using succinate relies on the dicarboxylate importer DctA, which is known to be regulated by the DcuSR two-component system in response to dicarboxylates, as well as by DctR (YhiF) in *E. coli* strains lacking ATP synthase activity (25–27).

Our previous studies of *Salmonella* translation elongation factor P (EF-P), which facilitates the ribosomal synthesis of difficult-to-translate polyproline/glycine motifs, employed Biolog Phenotype MicroArrays, revealing that strains lacking functional EF-P showed rapid growth under many nutrient-limited conditions (28–31). Here, we determine that the underlying cause of these phenotypes was that Biolog assays employ a base medium with succinate as the sole carbon source, a condition under which we find the *efp* mutant grows more rapidly than the wild type. We demonstrate that activation of RpoS, via the antiadaptor IraP or via the stringent response, triggers wild-type *Salmonella* to shut down import of dicarboxylates like succinate, fumarate, and malate. For reasons that remain unclear, *Salmonella* resumes import and growth on succinate medium after a typical lag of approximately 30 h. The severity of this lag differs between the vast majority of *Salmonella* and *E. coli* strains, and this difference appears to be due almost entirely to differences within the promoter of the dicarboxylate importer DctA. Furthermore, we find that trace amounts of proline or citrate can “license” early *Salmonella* growth on dicarboxylates. In the case of proline, this phenotype depends on the unusual polyfunctional enzyme/regulator PutA.

RESULTS

***Salmonella* delays its growth using dicarboxylic acids as a sole carbon source.**

Our previous work investigating the role of EF-P in *Salmonella* demonstrated that strains deficient in EF-P activity display a “hyperactive” metabolism relative to the wild type when grown under specific nutrient-limited conditions (28, 29). Biolog Phenotype MicroArrays (31) revealed that wild-type *Salmonella enterica* serovar Typhimurium strain 14028s failed to grow under several different nutrient conditions, whereas isogenic strains lacking functional EF-P grew rapidly. Through a series of further growth assays (described below), we determined that the underlying cause of this growth defect was due entirely to the fact that many of the varied growth conditions in the Biolog assay employ a base medium in which succinate provides the sole carbon source.

Earlier literature reported that many wild-type strains of *E. coli* display an extended lag when subcultured on succinate medium and that this growth delay was dependent on the sigma factor RpoS (22–24). Accordingly, we tested the growth of strain 14028s in minimal medium containing succinate as the sole carbon source. We found that the *efp* (EF-P) mutant, as well as an *rpoS* mutant, was able to grow readily in succinate with minimal lag (Fig. 1). In contrast, wild-type *Salmonella* exhibited an extended lag phase

FIG 1 *Salmonella* displays an extended lag phase using dicarboxylic acids as a sole carbon source, but *efp* and *rpoS* mutants do not. (A) Representative growth curve of *Salmonella* Typhimurium 14028s strains grown in MOPS minimal medium with 0.2% succinate as the sole carbon source. Data are the OD₆₀₀ values for 48 h from the time of inoculation. The data shown are representative of more than three biological replicates. An OD₆₀₀ of 0.1 is represented by a dashed line. (B) Graphs showing the average time in hours that wild-type (WT) or mutant *Salmonella* takes to reach an OD of 0.1 as an analog of the length of lag phase, using succinate as the sole carbon source. The data are the average result of at least three biological replicates, and error bars indicate 1 standard deviation. ">48h" indicates that the strain did not grow by 48 h. (C) Same as described for panel B but comparing the use of three different dicarboxylic acids as the sole carbon source.

where it failed to grow for over 30 h before initiating exponential (log) growth. The dicarboxylic acid transporter DctA was also necessary for growth, and an isogenic *dctA* deletion strain showed no sign of growth by 48 h.

We note that the wild-type log growth rate, when it finally resumed, was similar to the log growth rate of the *rpoS* mutant, suggesting that the growth delay was due to an extended lag phase upon subculturing into succinate medium and not due to slow growth on succinate *per se*. The length of this extended lag phase was remarkably consistent, typically between 30 and 32 h across dozens of experiments. Furthermore, colonies of *Salmonella* that grew on succinate medium after 30 h, when recultured, continued to display an extended lag in growth. Occasional colonies that did show rapid growth on succinate were found to have acquired spontaneous mutations in *RpoS*. Together, these results indicate that the bacteria that grew on succinate after 30 h did not arise from mutation but rather from a genetically programmed induction of succinate import.

DctA can also import other dicarboxylic acids, including malate and fumarate. We therefore tested whether the extended lag of wild-type *Salmonella* would also occur in media with fumarate and malate as the sole carbon source (Fig. 1C). We found that, similar to succinate, wild-type strain 14028s displayed a similarly extended lag in growth using either malate or fumarate, while the *efp* mutant grew significantly earlier. This suggests that the growth repression instigated by wild-type *Salmonella* is not specific to succinate but also occurs when subcultured into media with other dicarboxylic acids as the sole carbon source.

Many *Salmonella* but few *E. coli* strains delay growth using dicarboxylates.

Earlier literature suggested that the severity of the growth lag phenotype was much less in *E. coli* than what we observed in strain 14028s. To determine if this was indeed the case, or if it was merely a difference in growth conditions, the growth of *Salmonella* 14028s was compared to that of *E. coli* K-12 (strain BW25113). This revealed that while *E. coli* did display a lag in growth, this lag was much shorter than what was observed for *Salmonella* (Fig. 2A).

FIG 2 *Salmonella* grows significantly later than *E. coli* with succinate as the sole carbon source. Growth in MOPS minimal medium with 0.2% succinate as the sole carbon source. Growth was conducted in a Tecan Infinite M200 plate reader, and reads were taken every 15 min. (A) Representative growth curve. An OD₆₀₀ of 0.1 is represented by a dashed line. (B) Percentage of SGSC *Salmonella* (green line) or ECOR *E. coli* (blue line) strains that (on average) had surpassed an OD₆₀₀ of 0.1 by the indicated times postinoculation. The red line represents *Salmonella* strains but excludes the 14 tested strains of the SARC collection that do not belong to *S. enterica* subsp. *enterica*. (C) Overview of 105 *Salmonella* strains (SGSC collection) and 72 *E. coli* strains (ECOR collection). Each strain is plotted by the average time it takes to reach an OD of 0.1 (x axis) compared to the doubling time during growth from an OD of 0.1 to an OD of 0.2. All *Salmonella* strains are represented by black dots, and all *E. coli* strains are represented by red dots. Strains with at least one replicate (rep) that did not grow by 72 h are represented by triangles; values for these strains are the average result of the remaining replicates. Inset at top right, zoomed-out view to include outliers. (D) Average time in hours for strains to reach an OD of 0.1. Data are results for all 105 *Salmonella* strains compared to all 72 *E. coli* strains tested. A horizontal line indicates the median, boxes represent the 25th to 75th percentiles, and whiskers represent the 10th to 90th percentiles. An unpaired *t* test with Welch's correction indicates a *P* value of <0.0001 . (E) Same as described for panel D but comparing doubling time in hours. An unpaired *t* test with Welch's correction indicated a *P* value of <0.05 with all data points included or a *P* value of <0.0001 with slow-growing outliers excluded (doubling time, >5 h).

To determine whether the length of the dicarboxylate lag was a characteristic broadly shared among each of these two species or was limited to specific strains of *E. coli* and *Salmonella*, growth on succinate medium was assessed for all 105 nontyphoidal strains in the *Salmonella* Genetic Stock Centre (SGSC) collection, as well as all 72 strains of the *E. coli* Reference (ECOR) collection. Although there are some exceptions, the lag displayed by the vast majority of *E. coli* strains on succinate medium was

considerably shorter than that of most *Salmonella* strains (Fig. 2B to D). Once logarithmic growth was initiated, *Salmonella* also appeared to trend toward a slightly longer doubling time than the majority of *E. coli* strains (Fig. 2C and E).

Hypomorphic variants of *rpoS* accumulate readily in *E. coli* and *Salmonella* during laboratory passage, and there remained the possibility that many strains in the ECOR and SGSC collections acquired such mutations during their cultivation prior to being stored/archived. To ensure that the observed effects were not due to variations in RpoS activity, each strain was also screened for catalase activity as a surrogate indicator of having a functional RpoS. Regardless of catalase activity, the trend was maintained that *E. coli* strains in general showed a shorter lag phase than *Salmonella* when succinate was used as the sole carbon source (see Fig. S1 in the supplemental material).

IraP contributes to growth repression in dicarboxylate medium. RpoS is activated under specific conditions of stress or nutrient limitation, and its expression, synthesis, stability, and activity are regulated at multiple levels. Under ideal growth conditions, RpoS protein is destabilized through its interaction with RssB, an “adaptor” protein that promotes RpoS degradation via the ClpXP proteases. In response to specific stressors or nutrient limitations, one or more of the antiadaptor proteins IraP, RssC, and IraD can antagonize binding by RssB to alleviate RpoS degradation (14, 19, 20, 32). Systematic deletion of these antiadaptors revealed that IraP is a primary upstream factor involved in the dicarboxylate lag, and the rapid growth phenotype of an *iraP* mutant strain could be partially complemented by expressing IraP from its native promoter on a plasmid (Fig. 3). In contrast, deletion of the genes encoding the antiadaptors RssC and IraD had comparatively minor effects. These findings demonstrate that under the laboratory growth conditions we employ, IraP-mediated stabilization of RpoS plays a role in repressing *Salmonella*’s growth using succinate as the sole carbon source.

Proline and citrate override the lag observed in succinate medium. During these studies, it was observed that supplementation of our minimal medium with small amounts of LB medium (also known as lysogeny broth or Luria broth) would shorten or even eliminate the observed lag phase on succinate (Fig. S2). Given that the primary nutrient components in LB medium are peptides and amino acids, the effects of each amino acid and a few other carbon sources in the tricarboxylic acid (TCA) cycle were examined.

Unlike all other amino acids, we found that the addition of 0.005% proline to succinate medium could “override” the extended growth lag to enable rapid growth on succinate. The cells did not appear to simply consume the proline as a carbon source, since no other individual amino acid showed similar growth induction (Fig. S2D). Furthermore, although proline can be used by *Salmonella* as a carbon/nitrogen source, there was no discernible growth on 0.01% proline in the absence of succinate, suggesting that the levels we were providing were too small (Fig. S2E). A similar early-growth phenomenon could be invoked through the addition of small amounts of citrate. Growth on succinate in the presence of either proline or citrate occurred in a manner resembling diauxic growth, wherein growth would cease again following exhaustion of the proline or citrate (Fig. 4A and B). Using a *Salmonella relA spoT* double mutant that is unable to produce the secondary messenger ppGpp to trigger the stringent response, we also found that the stringent response is involved in sensing the depletion of either proline or citrate and resuming the growth lag. While the *Salmonella* stringent mutant displays a growth lag similar to that of the wild type when subcultured into succinate medium from an overnight culture in LB or M9 medium, it fails to instigate the rapid shutdown of growth upon exhaustion of proline or citrate and continues to grow instead of exhibiting the diauxic growth pattern (Fig. 4C and D).

Proline-mediated stimulation in growth requires the multifunctional proline utilization enzyme PutA. Proline has been shown to play a key, but unclear, role in *Salmonella*’s adaptation to life in an animal host. For both *E. coli* and *Salmonella*, proline can act as a compatible solute to regulate osmotic balance in the cells (33, 34). However, the key *Salmonella*-specific virulence factors encoded by *mgtA* and *mgtCBR* are controlled by short proline-rich peptides (*mgtL* and *mgtP*, respectively) that

FIG 3 Deletion of *IraP* results in early growth on succinate. Growth of *Salmonella* in MOPS minimal medium with 0.2% succinate as the sole carbon source. (A) The three known RssB antiadaptors were deleted from the *Salmonella* chromosome, and growth is shown along with that of an *rssB* mutant. Data are representative of at least three biological replicates. (B) Average time in hours for wild-type (WT) or mutant *Salmonella* strains to reach an OD of 0.1 as an analog of the length of lag phase with succinate as the sole carbon source. The data are the average result for at least three biological replicates, and error bars represent 1 standard deviation. (C) Plasmid expression of the *Salmonella iraP* gene partially complements the growth delay phenotype. (D) Same as described for panel B.

attenuate their operons in response to increased levels of uncharged tRNA_{pro} (35). Also, loss of EF-P, which is critical to ensure the smooth translation of proteins rich in proline, causes translational stalling that likely mimics the effects of proline limitation (30, 36, 37). Notably, *Salmonella* strains lacking EF-P are poorly virulent in animal models and appear to have difficulty adapting to an intracellular lifestyle, e.g., failing to produce typical *Salmonella*-induced filaments (sif's) when infecting host cells (29).

To explore the factors that might link proline availability to the rapid utilization of dicarboxylates as carbon sources, the roles of several proline-dependent regulatory systems were examined. While neither *mgtA* nor *mgtCBR* had an impact on proline's effect on the dicarboxylate growth lag (data not shown), we found that strains lacking PutA, an unusual polyfunctional proline utilization enzyme, were unresponsive to proline with respect to growth on succinate (Fig. 4 and B) (34, 38, 39).

Repression of dicarboxylate import accounts for growth lag. Not only does activation of RpoS upregulate several systems directly involved in mitigating cellular stress (e.g., catalase, import and synthesis of compatible solutes, and the production of glycogen, etc.), but it has also been shown to downregulate the expression of genes encoding enzymes in the TCA cycle (40–42). An analysis of *dctA* (encoding the primary dicarboxylate transporter) and *sdhA* (encoding succinate dehydrogenase for succinate metabolism) gene expression in the *Salmonella* Gene Expression Compendium (SalCom) database (43, 44) showed that they generally anticorrelated with the expression of RpoS-activated genes like *osmY* and correlated with growth phases where RpoS is inactive (e.g., *dctA* was downregulated in response to osmotic shock and in late stationary phase). We hypothesized that activation of the RpoS regulon may repress the expression of the *dctA* gene and thereby restrict *Salmonella* from taking up dicarboxylates such as succinate for consumption in response to stresses encountered within

FIG 4 Specific nutrients induce growth using succinate, and diauxic repression involves the stringent response. (A and C) Representative growth curves of *Salmonella* in MOPS minimal medium with 0.2% succinate as the primary carbon source and supplemented with 0.005% proline (+ Pro) or 0.005% citrate (+ Cit) where indicated. (B and D) Graphs showing the average time in hours for *Salmonella* to reach an OD of 0.1 as an analog of the length of lag phase with succinate as the sole carbon source. The data are the average result for at least three biological replicates, and error bars represent 1 standard deviation.

the host. To test if low *dctA* expression accounts for the *Salmonella* extended lag on dicarboxylates, we constitutively expressed *dctA* from a plasmid. Indeed, constitutive expression of *dctA* (but not of a similar *lacZ* control plasmid) eliminated the *Salmonella* lag in succinate medium (Fig. 5A and B).

The *E. coli* *dctA* promoter is sufficient to induce *Salmonella* growth using dicarboxylates. Given the differences between *Salmonella* and *E. coli* with respect to growth on succinate, we compared the nucleotide sequences upstream of *dctA* in these bacteria (Fig. S3). The two regions are largely similar (78% identity overall across 500 bp upstream), especially near the core promoter. However, there are notable regions of significant sequence difference that are specific and conserved for each species. A comparison of the *dctA* promoter of *Salmonella bongori* and of the various *S. enterica* subspecies (*enterica*, *houtenae*, *arizonae*, *diarizonae*, and *salamae*) and typhoidal isolates reveal that they are highly conserved in one another but distinct from the *dctA* promoters found in pathogenic and commensal *E. coli* strains (Fig. S3). This suggests that changes to the regulation of *dctA* may have occurred as, or shortly after, the two species diverged.

To examine whether the species-specific differences in the *dctA* promoter (P_{dctA}) played a role in the differences in how these species respond to succinate, the *E. coli* P_{dctA} was engineered into the *Salmonella* chromosome using an upstream chloramphenicol resistance cassette to select for successful recombination. Replacing the 500 bp upstream of the *Salmonella* *dctA* start codon with that of *E. coli* was sufficient to abolish *Salmonella*'s ability to repress its uptake of dicarboxylic acids, and this strain grew readily in succinate medium (Fig. 5C and D). As a control, using the same method to insert *Salmonella*'s native *dctA* promoter yielded no difference from wild-type *Salmonella*. Further reducing the swapped region to 200 bp maintained the growth phenotype, but swapping only 54 bp upstream of the *Salmonella* with that of *E. coli* (constituting the 5' untranslated region [UTR]) resulted in only a slight restoration of growth.

It is possible that *Salmonella* contains a factor to repress *dctA* expression that is not present in *E. coli*. We generated transcriptional fusion plasmids and tested the two

FIG 5 Expression of *dctA* induces growth using succinate. Growth of *Salmonella* in MOPS minimal medium with 0.2% succinate as the sole carbon source. (A) Overexpression of *dctA* from a plasmid. WT pLacZ and WT pDctA, wild-type *Salmonella* containing a pXG10sf plasmid encoding full-length *lacZ* and *dctA*, respectively, with expression driven by the constitutively active PLtet0-1 promoter. The *rpoS* mutant is shown for comparison. (B) Graph showing the average time in hours for wild-type (WT) or mutant *Salmonella* to reach an OD of 0.1 as an analog of the length of lag phase with succinate as the sole carbon source. The data are the average result for at least three biological replicates, and error bars represent 1 standard deviation. (C) Stretches of the *E. coli* *dctA* promoter (P_{dctA}) were inserted into the *Salmonella* chromosome, replacing the native *dctA* promoter. The lengths inserted (in base pairs counting back from the *dctA* start codon) are indicated. Replacement with *Salmonella*'s native *dctA* promoter controls for effects due to the insertion method [P_{dctA} (*Salm.*)]. Wild-type *Salmonella* and *E. coli* K-12 are shown for comparison. (D) Same as described for panel B.

dctA promoters when expressed in *E. coli* and found that the *dctA* promoter from *Salmonella* was expressed to a significantly lower degree than that of *E. coli* (Fig. S4).

Restricting dicarboxylate import does not influence survival in macrophage cell lines. To probe the question of why *Salmonella* may have acquired the trait of blocking dicarboxylate utilization and what evolutionary advantage it may gain by it, we considered that succinate levels increase significantly in activated macrophages, an environment in which *Salmonella* (but not *E. coli*) has adapted to survive effectively (5, 6, 45). In the *Salmonella*-containing vacuole, the pH reaches approximately 5.0 and the lower estimates reach pH 4.4, which is comparable to the acid dissociation constants of succinate ($pK_{a1,2} = 4.2, 5.6$) (46, 47). This suggests that in the acidified phagosome, succinate may become protonated and potentially act as a proton shuttle to acidify the bacterial cytoplasm. The ability of *Salmonella* to restrict its uptake of succinate could therefore provide a possible survival advantage in this environment, and data from SalCom suggests that *dctA* expression is repressed under *Salmonella* pathogenicity island 2 (SPI2)-inducing conditions and in macrophage (43, 44).

Constitutive overexpression of *dctA* but not *lacZ* led to decreased survival both in acidified succinate medium and in the human monocyte THP-1 cell line (Fig. S5). However, overexpression of *dctA* has been demonstrated to be toxic to *E. coli*, and we found that survival in acidified succinate was just as low for a *dctA* point mutant (N301A) that is defective for succinate transport (48). This suggested that the reduced survival was not due to dicarboxylate uptake but rather was an artifact of *dctA* overexpression to toxic levels. To bypass this artifact, we tested the *Salmonella* strain containing the chromosomal *dctA* promoter from *E. coli*, which grows readily in dicarboxylate medium yet does not constitutively overexpress *dctA* from a plasmid and so does not exhibit the associated toxic effects. Using this strain, we found no decrease in survival relative to that of wild-type *Salmonella* in acidified succinate medium or in human

(THP-1) or mouse (J774) macrophage cell lines (Fig. 6). Deletion of *dctA* or *iraP* genes also did not appear to significantly influence *Salmonella* survival in THP-1 macrophage in these short-term infection assays.

DISCUSSION

In this work, we explored a previously uncharacterized *Salmonella* phenotype whereby it restricts its ability to import dicarboxylates in an RpoS- and elongation factor P-dependent manner. Stationary-phase cells subcultured into medium with succinate, fumarate, or malate as the sole carbon source failed to grow for >30 h before resuming normal growth. The cause of this extended lag phase appears to largely reflect low expression of the *dctA* gene encoding a major dicarboxylate importer. The failure of strain 14028s to grow rapidly on succinate was not due to an inherent inability to utilize this carbon source (i.e., the strain does not lack the factors necessary to import or catabolize succinate), but rather, delayed growth was the result of a regulatory block that prevented the expression of the necessary succinate utilization systems. We believe that this phenotype may have been overlooked in prior studies on *Salmonella* metabolism, as many of these studies employed strain LT2, most isolates of which harbor a hypomorphic lesion in the gene encoding RpoS (49).

The “cue” that induces cells to suddenly begin rapid growth on dicarboxylates after 30 h remains unclear. Interestingly, an almost immediate exit from the extended lag phase could be triggered by the addition of low amounts of proline or citrate. Upon exhaustion of the available proline or citrate, growth would again cease in a process that required the synthesis of ppGpp. We considered the possibility that either proline or citrate is a limiting nutrient during growth on dicarboxylates (e.g., proline synthesis is shut down, and the cell becomes auxotrophic); however, several additional observations suggest that this is not the case. We note, for example, that growth is temporarily restored by either citrate or proline, and neither metabolite is known to be an intermediate in the synthesis of the other. Therefore, these two compounds are likely affecting growth through similar but independent routes. Furthermore, whereas wild-type *Salmonella* rapidly shuts down growth upon exhaustion of available proline or citrate, the stringent mutant continued to grow on succinate. This suggests that exhaustion of proline or citrate is not, in and of itself, limiting during growth in succinate medium but rather that proline or citrate exhaustion might act as an external cue that allows growth on dicarboxylates to start. In wild-type *Salmonella* strains, growth continues until these stimulating compounds are exhausted, at which point the production of ppGpp via the stringent response stimulates the shutdown of dicarboxylate utilization, possibly through its effects on RpoS.

We also noted that growth induction by proline required PutA, a polyfunctional membrane-bound enzyme that contains the two enzymatic activities required to oxidatively convert proline to glutamate, i.e., proline dehydrogenase (PRODH), which oxidizes proline to P5C, and P5C dehydrogenase (P5CDH), which is critical to convert P5C to glutamate. During the course of this conversion, PutA transfers the resulting electrons to ubiquinone (via PRODH), and NAD⁺ (via P5CDH). PutA also contains a DNA binding domain that controls, via repression, its own expression (*putA*) as well as that of the divergently transcribed *putP* gene, encoding the proline importer PutP. This finding implies that proline, *per se*, is not the proximal metabolite that regulates the use of dicarboxylates. Instead, the cue that triggers dicarboxylate use may be the PutA-mediated reduction of the quinone or NAD pools, PutA-mediated gene regulation (via its DNA binding domain), or the availability of glutamate/glutamine. We note, however, that neither glutamate or glutamine had the same effect on dicarboxylate growth as proline (see Fig. S2D in the supplemental material).

The model we derive from these data suggests that activation of RpoS leads to a rapid shutdown in the transporter that mediates the uptake and utilization of dicarboxylates (Fig. 7). Since the stringent response and ppGpp can impact the expression of *rpoS* and RssB antiadaptors, including *iraP*, the stringent response is likely the means

FIG 6 Replacement of the *Salmonella* *dctA* promoter does not influence survival in acidified succinate or macrophage. (A) Survival of *Salmonella* treated with LPM medium containing 0.2% succinate and acidified to pH 4.4. The wild type (WT) is compared to *Salmonella* with its chromosomal *dctA* promoter replaced with 500bp of the *E. coli* *dctA* promoter [P_{dctA} (E. coli)] and *Salmonella*'s native *dctA* promoter as a control [P_{dctA} (Salm)]. An *rpoS* mutant is shown as a positive control. The numbers of CFU recovered at 3 h were normalized to the numbers of input CFU at 0 h and are expressed as percent survival. Data are the average result across three biological replicates, and error bars represent 1 standard deviation. (B) Infection of THP-1 human macrophage comparing survival of wild-type *Salmonella* (WT) with various mutant strains and the *dctA* promoter swap strain. A *phoP* mutant is included as a positive control. Data shown are the log number of CFU recovered at 24 h postinfection and are the average result for three biological replicates. Error bars represent 1 standard deviation. (C) Same as described for panel B but showing the number of CFU recovered from mouse J774 macrophage.

by which *IraP* and *RpoS* mediate the shutdown of growth (14, 50–52). We also note the many curious links that have been found between regulation of genes essential for *Salmonella* host infection and the availability of proline, notably the attenuation peptides *MgtL* and *MgtP* and the essentiality of EF-P to alleviate translational stalling at proline-rich motifs. Our finding that the polyfunctional proline metabolic enzyme *PutA* is essential for the proline-mediated induction of growth on succinate leaves open the question of whether the inducing cue is due to changes in the ability of *PutA* to bind DNA (perhaps *PutA* directly represses *dctA* in *Salmonella*), alterations in cellular energetics via the production of either reduced quinones or NADH, or increases in the concentrations of cellular glutamate (although we note that addition of glutamate does not have the same effect as proline).

The difference in response of *Salmonella* and *E. coli* to dicarboxylic acids may offer important clues to identifying the evolutionary advantage conveyed by this

FIG 7 Model of the link between stress and dicarboxylate utilization in *Salmonella*. Under conditions of stress, RpoS (σ^S) levels are elevated due to the production of antiadaptors that block the degradation of RpoS by the RssB adaptor and ClpXP protease system. RpoS directs RNA polymerase (RNAP) to several promoters involved in stress protection as well as to a series of downstream regulatory genes (including small RNAs) that decrease the production of enzymes involved in the TCA cycle and other metabolic pathways. One of these downstream regulatory factors, yet to be identified, impinges on the expression of *dctA*. The presence of either citrate or proline is sufficient to alleviate the block on dicarboxylate utilization. The proline-mediated effect requires PutA, a polyfunctional enzyme that converts proline to glutamate with the concomitant production of reducing equivalents that are transferred to NAD^+ and ubiquinone (UQ) (figure created with BioRender). The factors that mediate the citrate-dependent effect remain unclear.

adaptation. The divergent evolutionary paths between *Escherichia* and *Salmonella*, which began over 100 million years ago, were fueled both by the acquisition of novel functions via horizontal gene transfer and extensive regulatory rewiring, often through changes in *cis*-regulatory elements (53–56). The differences in severity of their dicarboxylate lag, as evidenced by sampling a range of isolates in the SGSC and ECOR collections, appears to be due to differences in the 200-nucleotide region upstream of the *dctA* dicarboxylate import gene, including regions that lie upstream of the previously mapped 5' UTR (Fig. S3). While it remains possible that this factor could involve a small RNA, our finding that swapping just the 5' UTR of *dctA* (P_{dctA} 54) was insufficient to reverse growth repression suggests a protein factor acting on the promoter at the transcriptional level. RpoS likely impacts *dctA* expression via an intermediate and yet undetermined transcription factor. We employed the Virtual Footprint software (57) to predict possible transcription factor binding sites in the *dctA* promoter. The only transcription factor predicted to bind to *Salmonella* *dctA* but not *E. coli*, and within 200 bp of the start codon, was FhlA. However, FhlA's function as an activator (rather than a repressor), its requirement for formate, and its dependence on sigma-54 argue against a role in the dicarboxylate uptake phenotype observed in this work (58–60).

Since many of the traits that *Salmonella* has acquired since its divergence from *E. coli* are related to its pathogenic lifestyle, it follows that this phenotype may reflect a situation that *Salmonella* encounters during infection of a host. The recent finding that succinate accumulates to high levels in activated macrophage suggests that *Salmonella*'s intracellular survival may represent the crucial selective environment that has led to the *dctA* repression phenotype (45). It is conceivable that *Salmonella* recognizes the succinate produced by activated macrophage and restricts its uptake of dicarboxylates in response. Our examination of *Salmonella* strains that are impaired in their regulation of dicarboxylate uptake identified no survival defect in acidified succinate or in macrophage cell lines. However, it remains possible that this repression phenotype is related to other aspects of the *Salmonella* virulence cycle beyond short-term survival in phagocytes.

We did note a few *Salmonella* strains that grew early on succinate despite having a catalase-positive (and presumably *rpoS*⁺) phenotype. In all instances where multiple strains from the same *Salmonella enterica* serovar were tested, at least one exhibited an extended lag phase (Table S1). Thus, the earlier growth does not appear to be a trait of particular serovars but rather may reflect individual strains having lost (or never acquired) the delayed growth phenotype. This could occur by mutations in genes other than *rpoS*, such as *efp* or *iraP*, that permit early growth in dicarboxylic acids. Other exceptions include multiple *E. coli* strains that show an extended lag in their growth using succinate. While it is possible that these strains have mutations in genes required for the uptake of succinate (such as *dctA* or *dcuSR*), they may reflect genuine variation in how *E. coli* strains respond to dicarboxylates.

Finally, our findings have important implications for the interpretation of previous metabolic studies in commonly used laboratory strains. For example, it is important to consider that the Biolog assays primarily use succinate as the carbon source under many conditions (28, 29, 31). Thus, what we previously assumed to be improved growth of the *efp* mutant under a variety of different conditions in fact resulted solely from its ability to rapidly utilize dicarboxylate medium. We also note that a large number of metabolic studies were carried out with the commonly used *Salmonella* Typhimurium strain LT2, which is a known *rpoS* mutant (49). Because of this, studies using the LT2 strain would behave quite differently in Biolog assays than the other commonly used “wild-type” *Salmonella* strains, SL1344 (of which many isolates are auxotrophic for histidine) and 14028s.

Many outstanding questions remain to be answered in future research. What is the key regulator of *dctA* expression, and what is its binding site in the *dctA* promoter? How do proline, citrate, and ppGpp influence it? Do other transporters take over dicarboxylate import under different conditions such as low pH? Nevertheless, this study identifies a phenotype diverging between *Salmonella* and *E. coli*, begins to identify some components of the underlying mechanisms involved, and highlights some of the reasons why such a phenotype may have been overlooked in the past.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains, plasmids, and primers are listed in Tables S2 to S4 in the supplemental material. As described previously, lambda red recombination (28, 61) and subsequent P22 phage transduction (62) were used to generate all of the gene knockout mutants in *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*S. Typhimurium*) strain 14028s. *E. coli* was from the Keio collection K-12 BW25113 strain background (63). To sample the genetic diversity of *Salmonella* and *E. coli* isolates, the *Salmonella* genetic stock center (SGSC) SARA (64), SARB (65), and SARC (66) collections were employed and compared to the *E. coli* reference (ECOR) collection (67).

The full-length *dctA* open reading frame (ORF) was expressed from the pXG10sf plasmid under the control of the constitutively active PLtetO-1 promoter (68, 69). The *IraP* complementation plasmid was generated by inserting the *iraP* ORF and the upstream 300 bp into pXG10sf. For promoter expression, the *dctA* promoter (500 bp upstream of the *dctA* start codon) was inserted into pXG10sf to drive expression of superfolder green fluorescent protein (GFP) (69, 70). To generate the chromosomal *dctA* promoter swap strain, 500 bp upstream of the *E. coli* *dctA* start codon, along with a chloramphenicol resistance cassette for selection, was inserted into the corresponding location of the *Salmonella* chromosome.

Growth using dicarboxylates as a sole carbon source. Overnight LB cultures inoculated from single colonies were resuspended in MOPS (morpholinepropanesulfonic acid) minimal medium with no carbon source to an optical density at 600 nm (OD₆₀₀) of approximately 1.75. This suspension was used to inoculate (1/200 dilution) MOPS minimal medium containing 0.2% carbon source (succinate unless otherwise indicated). Growth was conducted in a Tecan Infinite M200 plate reader at 37°C with shaking, and the OD₆₀₀ was read every 15 min. For salts and hydrates of carbon sources, the final concentration reflects the percent of the carbon source itself (e.g., 0.2% succinate was made as 0.47% sodium succinate dibasic hexahydrate).

For screens of the SGSC and ECOR collections, 47 strains were assessed in duplicate per run in a 96-well plate. Wild-type and *rpoS* mutant *Salmonella* bacteria were included on every plate as quality controls. Each strain was tested on at least three separate days.

Catalase assay. For each replicate of the SGSC and ECOR collection screens, each strain was tested for catalase activity as an analog for RpoS function (71). In parallel to the LB overnight cultures used as the inoculum, 10 μ l of each culture was spotted onto an LB plate. The next day, the spots were tested for catalase activity by the addition of 10 μ l hydrogen peroxide. Bubbling was scored in comparison to wild-type (catalase-positive) and *rpoS* mutant (catalase-negative) *Salmonella*.

Acid survival. LPM medium was made as described previously (72), and succinate was added to either 0.2% or 0.4%, as indicated in the figures. The pH of the medium was then adjusted to 4.4. LB overnight cultures were resuspended to an OD of 0.1 in acidified medium and incubated in a 37°C water bath. At time points, samples were taken, serially diluted, and plated for CFU counting.

Intramacrophage survival. The THP-1 human monocyte cell line and the J774 mouse macrophage cell line were maintained in RPMI 1640 medium (with L-glutamine) supplemented with 10% fetal bovine serum (FBS) and 1% Glutamax and grown at 37°C and 5% CO₂. For infection assays, THP-1 cells were seeded in 96-well plates at 50,000 per well, with 50 nM PMA (phorbol 12-myristate 13-acetate) added to the medium to induce differentiation to adherent macrophage. After 48 h, the medium was replaced with normal growth medium (no PMA) overnight. For infections with J774 macrophage, the cells were seeded in 96-well plates at 50,000 per well overnight. *Salmonella* bacteria in RPMI 1640 were added to seeded cells at a multiplicity of infection (MOI) of approximately 20 bacteria to 1 macrophage and centrifuged for 10 min at 1,000 rpm for maximum cell contact. After centrifugation, the plate was placed at 37°C (5% CO₂), and this was called time zero. After 30 min, nonadherent *Salmonella* cells were washed off by three washes with phosphate-buffered saline (PBS), followed by replacement with fresh medium containing 100 µg/ml gentamicin to kill extracellular *Salmonella*. At 2 h, the medium was replaced with medium containing gentamicin at 10 µg/ml. At time points, intracellular bacteria were recovered using PBS containing 1% Triton X-100 and vigorous pipetting. Samples were serially diluted, and five 10-µl spots were plated for CFU counting. Each sample included three separate wells as technical replicates (a total of 15 10-µl spots counted per biological replicate).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.03 MB.

SUPPLEMENTAL FILE 2, PDF file, 1.1 MB.

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