

Control of pili synthesis and putrescine homeostasis in *Escherichia coli*

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eLife Assessment

This **valuable** study presents an interesting analysis of the role of the polyamine precursor putrescine in the pili-dependent surface motility of a laboratory strain of *Escherichia coli*. The genetic data **convincingly** shows this role, yet without putrescine measurements, the evidence remains **incomplete**. This work will be of interest to biomedical scientists studying uropathogenic bacteria, and those studying bacterial polyamine function.

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Abstract

Summary

Polyamines are biologically ubiquitous cations that bind to nucleic acids, ribosomes, and phospholipids and, thereby, modulate numerous processes, including surface motility in *Escherichia coli*. We characterized the metabolic and transcription factors that contribute to polyamine-dependent control of surface motility. Genetic analysis showed that surface motility required type 1 pili and the simultaneous presence of two independent putrescine anabolic pathways and that putrescine transport and catabolism modulated surface motility. The results of an immunological assay for FimA—the major pili subunit, reverse transcription quantitative PCR of *fimA*, and transmission electron microscopy confirmed that pili synthesis required putrescine. RNAseq analysis of a parental and putrescine-deficient mutant and a follow-up genetic analysis suggested that H-NS was the putrescine-responsive regulator of pili synthesis and surface motility. The RNAseq results also showed that low putrescine increased transcripts for genes of arginine synthesis and transport systems for magnesium and phosphate, and decreased transcripts of genes for oxidative energy metabolism and iron transport. We conclude that (a) putrescine controls pili synthesis and surface motility possibly via the transcriptional regulator H-NS, and (b) a complex homeostatic network, which can rewire metabolism, controls putrescine concentrations. During a urinary tract infection, the polyamine putrescine is present in urine and pili are required for the binding

of bacteria to the bladder epithelium. Because of its control of pili synthesis and energy metabolism, putrescine is likely to contribute to the establishment and maintenance of urinary tract infections associated with uropathogenic *Escherichia coli*.

Introduction

Polyamines are flexible aliphatic cations that are found in virtually all organisms. *E. coli* contains putrescine (1,4-diaminobutane) and lesser amounts of spermidine (N-(3-aminopropyl)-1,4-diaminobutane), and cadaverine (1,5-diaminopentane) (1, 2). Both the pathways and enzymes of polyamine synthesis are redundant (Fig. 1). Strains that lack eight of these genes do not contain detectable polyamines, grow slowly aerobically, and do not grow anaerobically (3). Polyamines interact with nucleic acids and phospholipids, can affect chromosome and ribosome structure, ribosome-mRNA interactions, and protein and nucleic acid elongation rates (4). Polyamines modulate, but are not required for, protein synthesis (5) and expression of hundreds of genes (6). A major mechanism of polyamine-dependent regulation is translational control of genes for 18 major transcription regulators: four of the seven σ factors – σ^{18} (FecI), σ^{24} (RpoE), σ^{38} (RpoS), and σ^{54} (RpoN); three histone-like DNA-binding proteins (Fis, H-NS, and StpA); and CpxR, Cya, Cra, and UvrY (5, 6).

Polyamines control flagella-dependent surface motility of *Proteus mirabilis* and pili-dependent surface motility (PDSM) of *E. coli*: the latter has been reported to require the polyamine spermidine (7, 8). While reconstructing putrescine-deficient strains to characterize the basis for the anaerobic growth defect, we noticed that a mutant unable to synthesize spermidine moved normally, while mutants lacking enzymes of putrescine synthesis moved poorly. We characterized the role of polyamines on *E. coli* surface motility and pili synthesis by genetic analysis, ELISA assays, RT-qPCR, electron microscopy, and RNAseq. We showed that PDSM and pili synthesis required two independent pathways of putrescine synthesis, but did not require spermidine, and was modulated by putrescine transport and catabolism. In addition to confirming that putrescine controlled pili synthesis, results from an RNAseq analysis identified regulators that mediated putrescine-dependent control, and, unexpectedly, suggested a putrescine homeostatic network that rewires metabolism to maintain intracellular putrescine.

Results

Pili-dependent surface motility (PDSM) of W3110

While characterizing mutants lacking putrescine synthetic enzymes, we found that some strains were defective in surface motility. Because any single laboratory strain may have properties that result from unintended laboratory evolution, we assessed nine strains for their surface motility pattern. Five strains covered a plate in 12-18 hours, while four did not traverse a plate in 36 hours. The slow-moving strains generated genetically stable fast-moving variants which suggests that the former were ancestral. Our lab strain of W3110 moved slowly, and among the slow movers showed the least variability in spreading diameter and generated fewer fast-moving variants. Its outward movement formed concentric rings, with alternating phases of movement and consolidation/growth (Fig. 2A). Deletion of *fimA*, which codes for the major type 1 pili subunit, abolished the oscillatory pattern and reduced, but did not eliminate, surface motility, but deletion of *fliC* which encodes the major flagellum subunit did not affect surface motility (Fig. 2A). Electron microscopy confirmed that W3110-LR isolated from surface motility plates possessed pili (Fig. 2B). None of >500 cells expressed flagella. A mixed population of elongated (3-4 μ m) and non-elongated (<2 μ m) cells were visible, and pili were mostly associated with elongated cells. Furthermore, W3110 surface motility requires glucose (9) which should prevent flagella

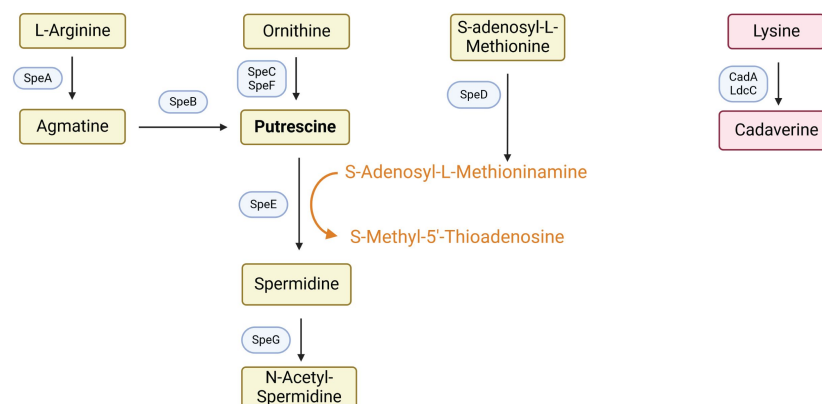


Figure 1
Pathways and enzymes of polyamine synthesis.

synthesis via inhibition of cyclic AMP synthesis (10). We confirmed that glucose controlled flagella synthesis in W3110 by its suppression of swimming motility (Fig 2C). A W3110-LR derivative with deletions of both *fliC* and *fimA* still exhibited outward movement on a surface motility plate (Fig 2A), but electron microscopy showed the absence of appendages (Fig. 2C). In summary, genetics, electron microscopy, and a glucose requirement show that W3110 surface motility requires pili.

PDSM required two independent putrescine synthesis pathways

We examined PDSM requirements for polyamine anabolic pathways. A major putrescine biosynthetic pathway is ornithine decarboxylation either by the constitutive SpeC or the low-pH inducible SpeF. The Δ *speC* and Δ *speF* mutants moved as well as the parental strain, but a Δ *speC* Δ *speF* double mutant moved less well (Figure 3). SpeA (arginine decarboxylase) and SpeB (agmatinase) catalyze an alternate two-step putrescine synthetic pathway (Fig 1) (11): deletion of either gene reduced surface motility (Figure 3). The *DspeA*, *DspeB*, and *DspeC* *DspeF* strains grew as well as the parental strain (Supplemental Figure 1A) and had normal swimming motility (Supplemental Figure 1B). A *ΔspeE* mutant, which lacks the enzyme for spermidine synthesis, moved on a surface as well as the parental strain (Fig 3).

Supplemental putrescine at 1 mM restored surface motility of a *speB* mutant (Figure 4A) and a *speA* mutant (not shown). Spermidine did not restore wild type movement and, also, caused an unusual pattern of movement in the parental strain (Figure 4B). These exogenous concentrations of putrescine and spermidine had no effect on growth rates in liquid motility medium (not shown). In summary, PDSM required the simultaneous presence of two independent pathways of putrescine synthesis but did not require spermidine.

Loss of putrescine transport systems affected surface motility

Putrescine transport may maintain an appropriate intracellular putrescine concentration, because *E. coli* secretes putrescine (12). *E. coli* has several putrescine transport systems (13, 14). Mutants lacking the PlaP and PotF putrescine transporters had 50% and 40% reduced surface motility, respectively, and lost the concentric ring pattern (Figure 5A). Loss of the PotE and PuuP transporters affected neither the movement diameter (Figure 5B) nor concentric ring formation (Figure 5A). We note that *potE* and *plaP* are more highly expressed than *potE* and *puuP* (85, 64, 17, and 6 counts per million transcripts, respectively) for W3110 grown in liquid motility medium (15). We conclude that extracellular putrescine and its transport contribute to PDSM.

Putrescine catabolism affected surface motility

Because putrescine catabolism modulates intracellular putrescine concentrations (16, 17), we examined PDSM in putrescine catabolic mutants. The major putrescine catabolic pathway is initiated with putrescine glutamylation (reactions 6-9, and 4-5, Fig 6A), and a second pathway initiates with putrescine deamination (reactions 1-5, Fig 6A) (17). Deletion of either *puuA* or *pata* which code for the first enzymes of their respective pathways did not affect surface motility (Fig 6B). A double mutant moved less well which was unexpected because the proposed higher intracellular putrescine should have stimulated PDSM. Consistent with the stimulatory effect of putrescine, loss of *puuR* which codes for the repressor of PuuA-initiated pathway genes (17), impaired PDSM (Fig 6B). One possible explanation to reconcile these seemingly contradictory results is that an optimal polyamine concentration stimulates PDSM, and a high concentration is inhibitory. Experiments that are described in the next section test this possibility.

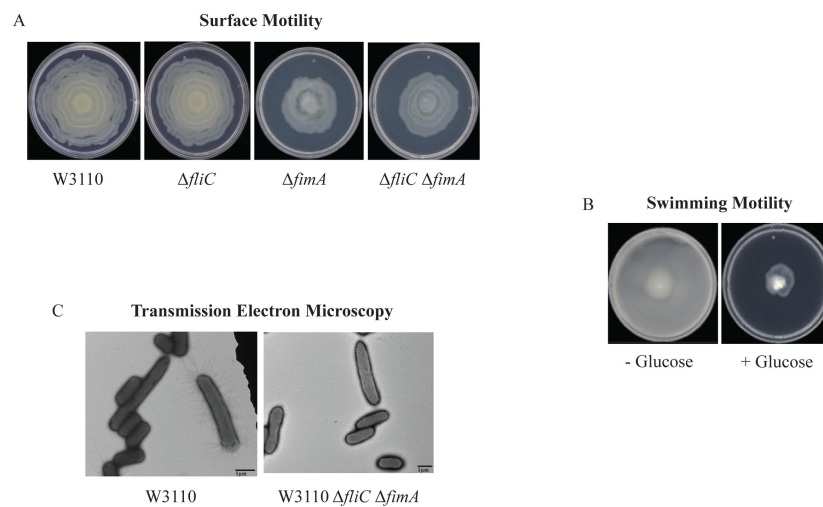


Figure 2.

Surface motility of W3110-LR. (A) Surface motility of parental and derivative strains lacking the major subunits of the flagella (FliC), pili (FimA), or both. (B) Swimming motility with and without 0.5% glucose. (C) TEM images of cells of W3110-LR and W3110-LR $\Delta fliC \Delta fimA$ taken from the movement's edge directly from surface motility plates.

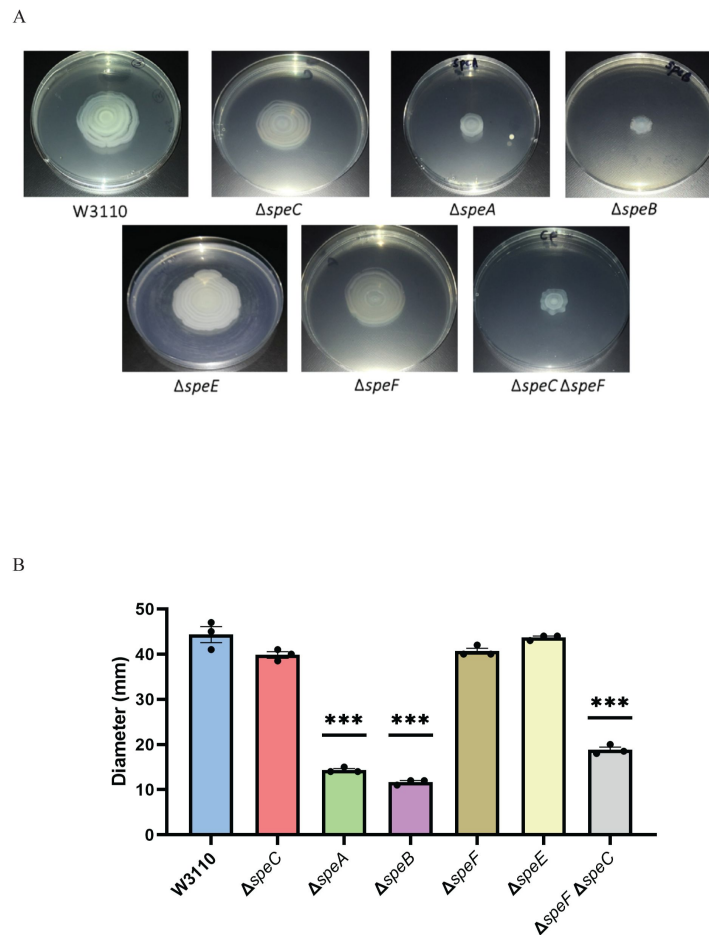


Figure 3.

Genetics of surface motility. (A) PDSM of mutants with defects in polyamine anabolic genes. (B) Diameter of surface movement of polyamine mutants after 36 hours. Error bars represent standard deviations for three independent replicates. Statistical analysis was performed using the Dunnett test of significance: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. All assays were performed in triplicate and representative images are shown. In this figure the $\Delta speB$ mutant was IM26.

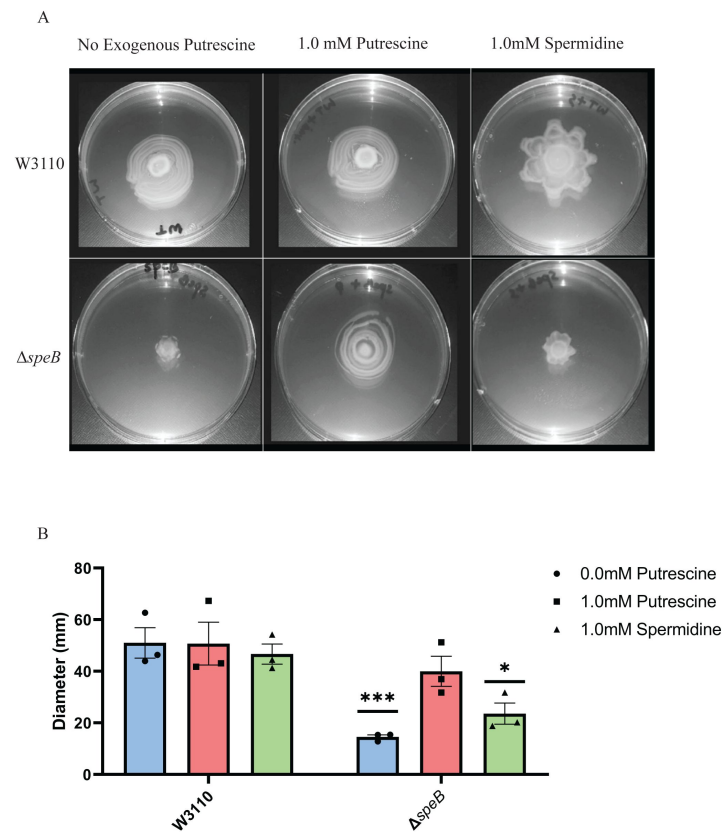


Figure 4

Nutritional supplementation of $\Delta speB$ mutant. (A) Putrescine and spermidine supplementation of wild type and $\Delta speB$ strains. (B) Diameter of surface movement of polyamine mutants after 36 hours. Error bars represent standard deviations for three independent replicates. Statistical analysis was performed using the Sadik test of significance: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. All assays were performed in triplicates and representative images are shown. In this figure the $\Delta speB$ mutant was IM26.

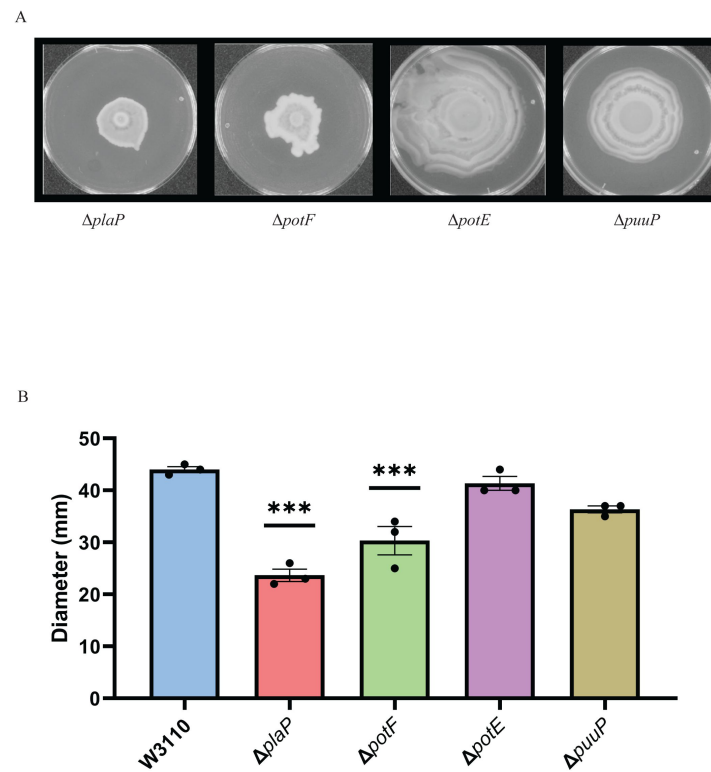


Figure 5

Putrescine transport and surface motility. (A) Representative images of surface motility for strains defective in putrescine transport genes. (B) Diameter of surface movement of polyamine mutants after 36 hours. Error bars represent standard deviations for three independent replicates. Statistical analysis was performed using the Dunnett test of significance: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. All assays were performed in triplicate and representative images are shown.

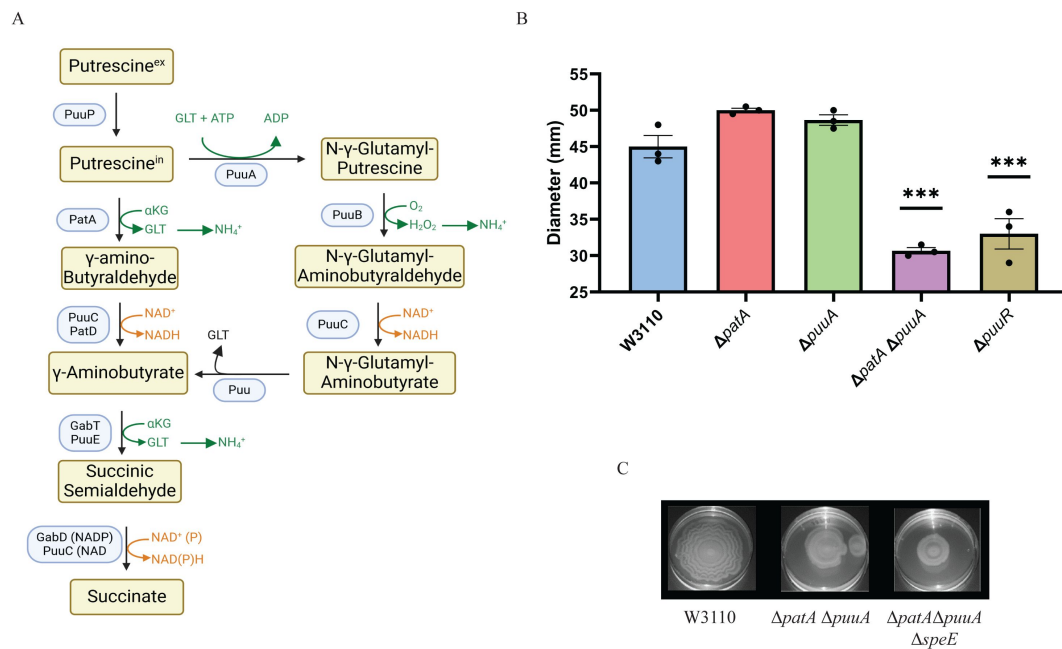


Figure 6

Putrescine catabolism and surface motility. (A) Pathways and enzymes of putrescine catabolism. (B) Motility diameter of putrescine catabolic mutants after 36 hours. Error bars represent standard deviations for three independent replicates. Statistical analysis was performed using the Dunnett test of significance: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. All assays were performed in triplicates and representative images are shown. (C) Effect of loss of *speE* on the *patA puuA* catabolic double mutant.

High putrescine reduced expression of pili genes

High putrescine inhibits translation of some mRNAs, and high spermidine, a product of putrescine metabolism, inhibits growth (18, 19). If the phenotype of the $\Delta patA \Delta puuA$ double mutant results from spermidine toxicity, then loss of spermidine synthase (SpeE) should reverse the phenotype. This prediction was not met which argues against spermidine toxicity (Fig 6C). For the *speB* mutant, 1 mM putrescine stimulated PDSM, and 4 mM putrescine was slightly inhibitory but eliminated the ring pattern (Fig 7AB). The concentric ring pattern was observed with 1 mM, but not 4 mM putrescine. Indirect enzyme linked immunosorbent assays (ELISAs) against FimA showed that the level of FimA in the *speB* mutant at 1 mM putrescine was higher than with 4 mM putrescine (Fig. 7C). Variation of supplemental putrescine did not affect FimA in the parental strain (Fig. 7C).

To test transcriptional regulation of pili expression by putrescine, we examined transcript levels by RT-qPCR. Optimal *fimA* transcription occurred at 1 mM putrescine in the *speB* mutant (Fig 7D). Transmission electron microscopy (TEM) showed that pili expression was highest for the *speB* mutant at 1 mM putrescine (Fig. 8), moderate at 0.1- and 4-mM putrescine, and undetectable without putrescine. The cells grown with 4 mM putrescine were shorter and thinner, which suggests a stress, possibly mimicking osmotic stress. From these results and those in the previous section, we conclude that pili expression required an optimal putrescine concentration.

RNAseq analysis identified H-NS as a possible mediator of putrescine-dependent control

To determine whether putrescine affects transcription of genes whose products control *fim* operon expression (20), we performed RNAseq analysis of the parental and *speB* strains grown with and without 1 mM putrescine. The transcriptomes of the parental strain both with and without putrescine and the *speB* mutant with 1 mM putrescine were highly similar with R^2 values of 0.950 and 0.954, respectively, based on expression graphs comparing the transcriptomes of the parental strain in both conditions to the *speB* mutant strain (Supplemental Figure 2). Compared to the transcriptome of the *speB* mutant grown without putrescine, the transcriptomes of the parental strain without putrescine ($R^2 = 0.796$), the parental with 1 mM putrescine ($R^2 = 0.786$), and the *speB* mutant with 1 mM putrescine ($R^2 = 0.820$) differed substantially. A multidimensional scaling plot and a plot of the 100 most variable genes further visualize these interactions (Fig 9A and 9B).

When compared to the parental strain, the *speB* mutant grown without putrescine had 310 downregulated genes and 159 upregulated genes with at least four-fold differential expression and $FDR < 0.05$. Transcripts for the putrescine-responsive *puuAP* and *puuDRCBE* operons, which specify genes of the major putrescine catabolic pathway, were reduced from 1.6- to 14-fold ($FDR \leq 0.02$) in the *speB* mutant (Supplemental Table 1), which implies lower intracellular putrescine. Deletion of *speB* reduced transcripts for genes of the *fimA* operon and *fimE*, but not of *fimB* (Fig 10A). Furthermore, loss of *speB* affected some of the known positive effectors that control *fim* operon expression which include Fis, H-NS, IHF, Lrp, and QseB (21). Transcripts from *hns* were reduced, transcripts from *ihfA* and *ihfB* were not affected, and transcripts from *fis*, *lrp*, and *qseB* were increased when *speB* was deleted (Fig 10B). Loss of *speB* also increased transcripts from *lrhA* which codes for a positive effector of *fimE*. Loss of several of these regulators eliminated PDSM which was expected (Fig 10C). H-NS may mediate putrescine-dependent control of pili synthesis. However, given the numerous effects of loss of *speB*, a generalized dysregulation of several major transcription factors may also produce a less-than-optimal constellation of regulators.

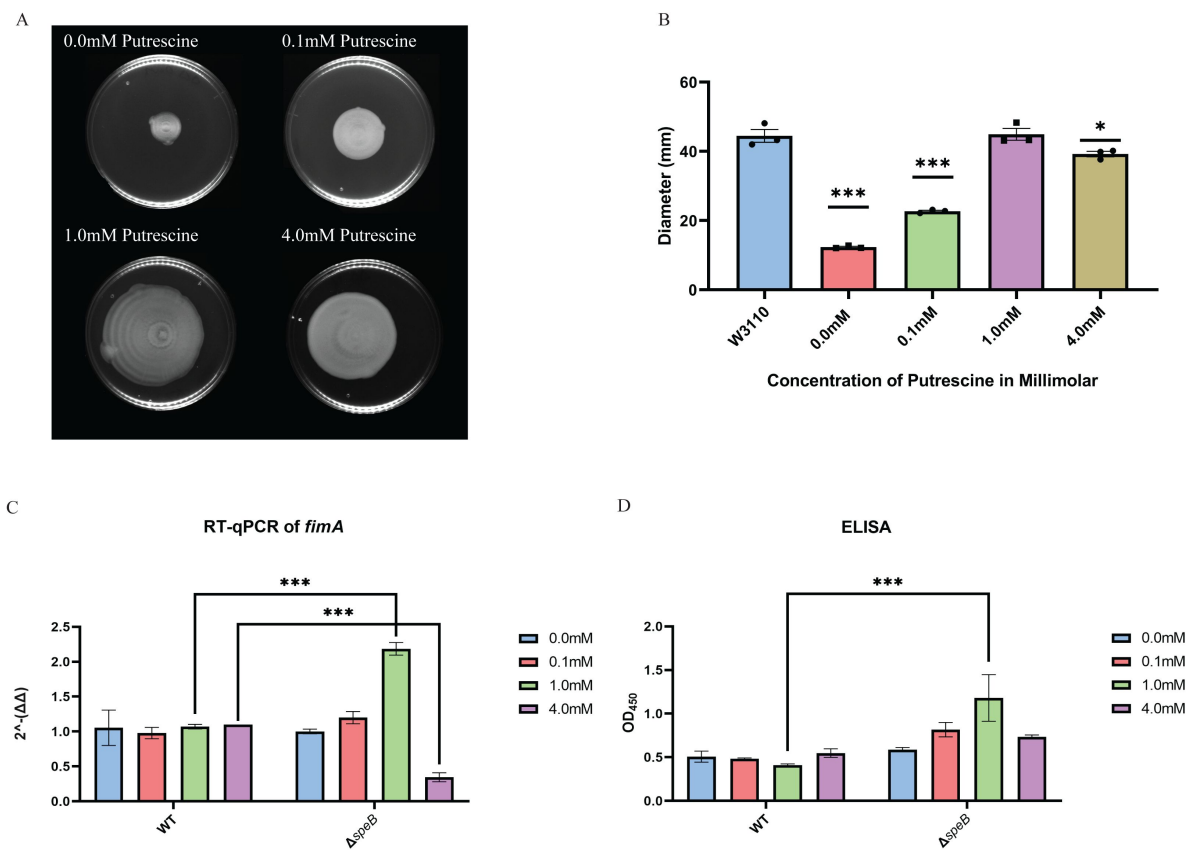


Figure 7

Surface motility and pili expression in W3110 and $\Delta speB$. (A) W3110 $\Delta speB$ surface motility with 0.0, 0.1, 1.0, and 4.0 mM exogenous putrescine. All assays were performed in triplicate. Representative images are shown. (B) Average diameter of 3 separate surface motility plates. The parental strain without putrescine is shown for reference. Significance was determined by comparing the diameters of the $\Delta speB$ mutants in the different concentrations of putrescine compared to the parental W3110. One way ANOVA was used with Dunnett hypothesis testing to determine p values, ***, $p < 0.001$. (C) Reverse transcriptase-quantitative PCR using primers targeting the *fimA* gene from cells grown in 0.0, 0.1, 1.0, and 4.0 mM of supplemented putrescine. Double deltas were generated by normalizing the parental and the $\Delta speB$ mutant RNA libraries to *rpoD* then comparing *fimA* expression. Two-way ANOVA was used to determine significance followed by Sidak hypothesis testing. ***, $p < 0.001$. (D) Enzyme linked immunosorbent assays using antibodies targeting pili (FimA) from cells grown with 0.0, 0.1, 1.0, and 4.0 mM of supplemented putrescine. Two-way ANOVA was used to determine significance followed by FDR adjusting. ***, $p < 0.001$. In this figure the $\Delta speB$ mutant was J15.

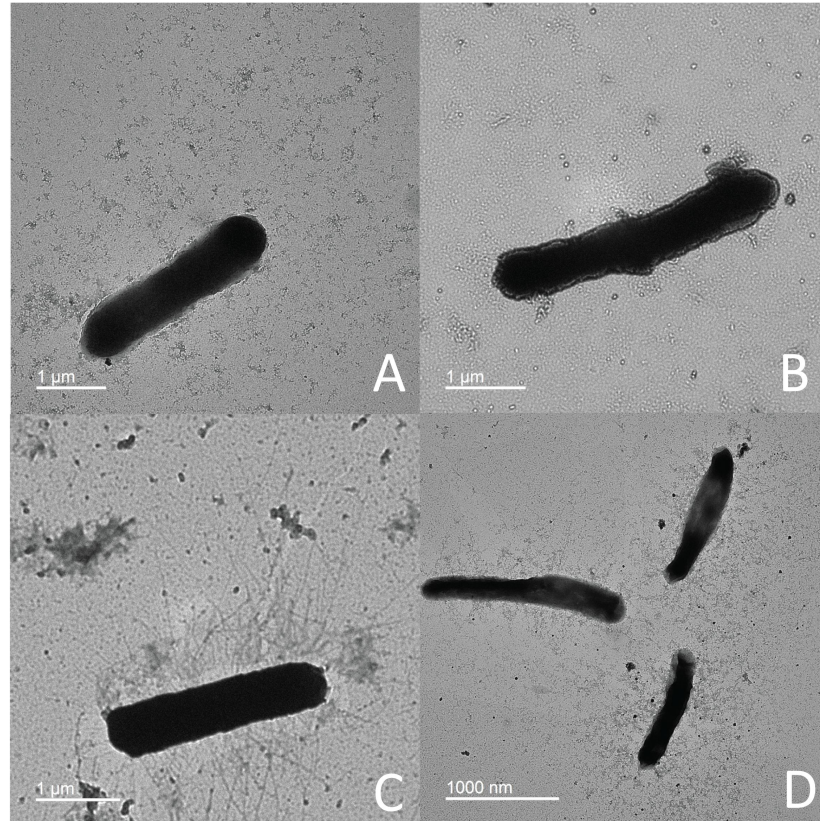


Figure 8

Transmission electron micrographs of $\Delta speB$ mutant cells after surface motility. Representative images are shown and cells were removed from motility plates with the following exogenous putrescine concentrations: (A) none, (B) 0.1 mM, (C) 1.0 mM, and (D) 4.0 mM. No pili were observed with 0 and 0.1 mM putrescine. Optimal pili production was observed with 1.0 mM putrescine. Shorter and less pili were observed with 4.0 mM. The arrows in (D) point to apparent pili fragments. The bar represents one micron. In this figure the $\Delta speB$ mutant was J15.

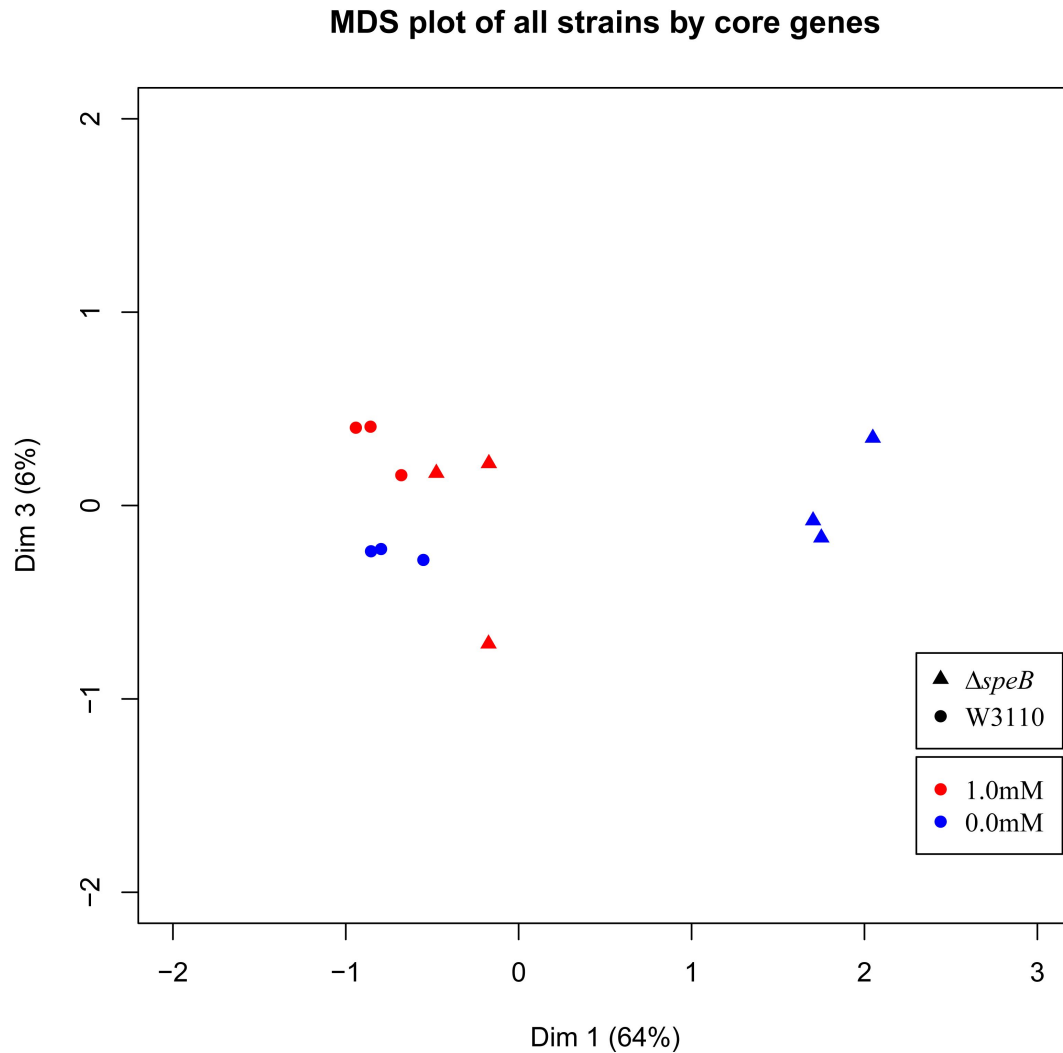


Figure 9

Visual representation of the results from transcriptomic sequencing of W3110 and the *ΔspeB* mutant's gene expression in media with and without 1.0 mM putrescine. (A) Multidimensional scaling plot of the parental and *ΔspeB* mutant transcriptomes. When grown with 1.0 mM putrescine (red), the *ΔspeB* mutant transcriptome (triangles) are nearly identical to the parental transcriptomes when grown without and with putrescine (blue and red circles, respectively). When grown without putrescine, the *ΔspeB* mutant transcriptome (blue triangles) is greatly skewed from transcriptomes of the parental strain and the *ΔspeB* mutant grown with putrescine. (B) Heatmap of the top 100 most variable genes further demonstrates the distinctiveness of the *ΔspeB* mutant grown without putrescine and the similarities of the *ΔspeB* mutant transcriptome when grown with 1.0 mM putrescine supplementation and the parental grown with or without putrescine. In this figure the *ΔspeB* mutant was J15.

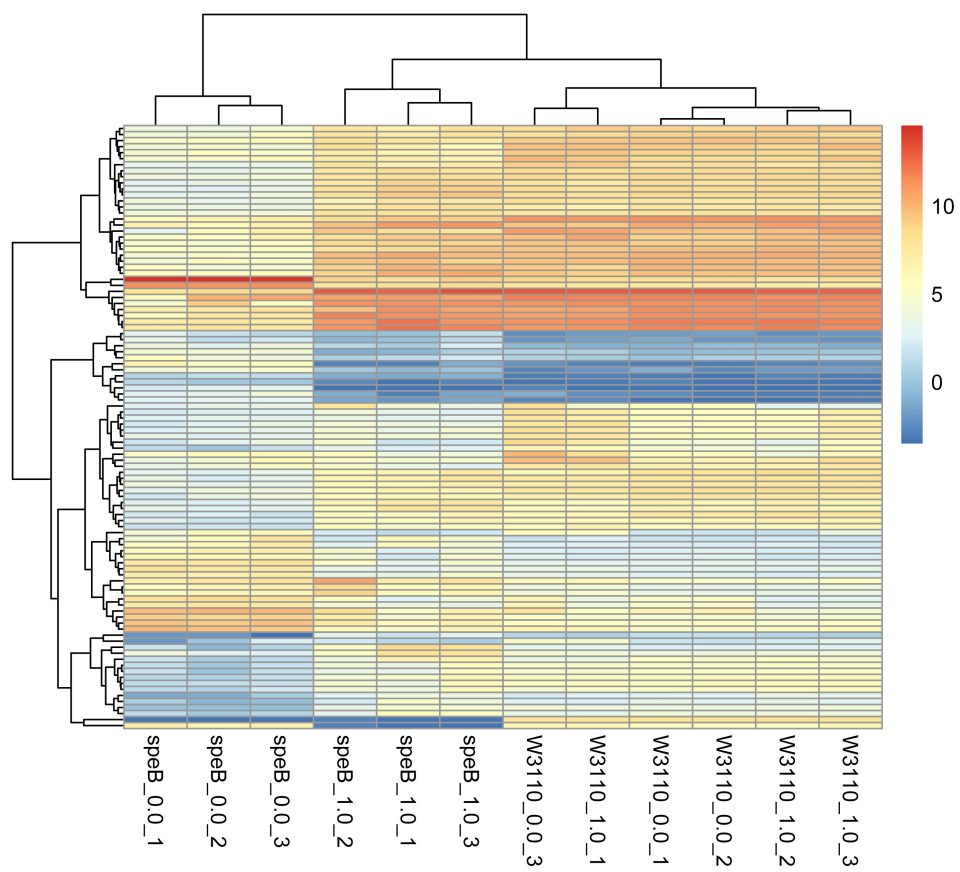


Figure 9 (continued)

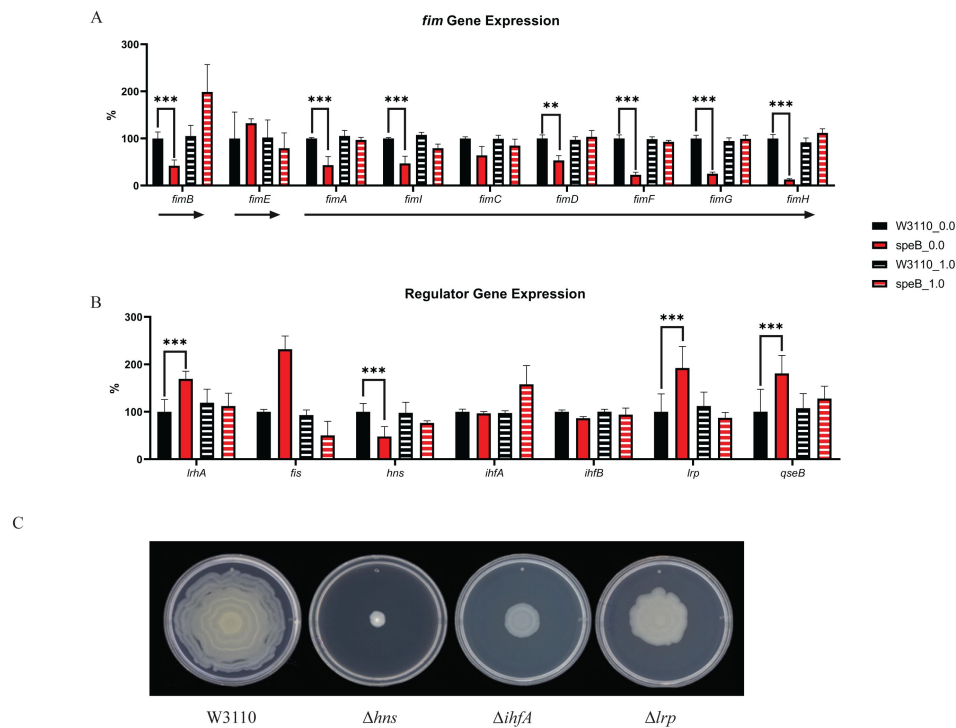


Figure 10

Transcriptomic sequencing of genes for the *fim* region and regulators that control *fim* gene expression. (A) Expression of the *fim* genes in the parental W3110 and the *speB* mutant with and without putrescine supplementation. Values were calculated by dividing the individual replicates' counts per million (CPM) value for each gene by the mean CPM of that gene in W3110 grown without putrescine and multiplying by 100 to yield the percent expression. Arrows below the genes signify the known operons: *fimB* and *fimE* belong to single gene operons, while *fimAICDFGH* belong to one operon. One way ANOVA was used to determine significance using Dunnett hypothesis testing. **, $p < 0.01$; ***, $p < 0.001$. (B) Expression of some regulators known to affect *fim* gene expression. Values were calculated as described in (A). ***, $p < 0.001$. (C) Surface motility of W3110 and three regulator mutants (Δhns , $\Delta ihfA$, and Δlrp). A gene found to be significantly different by this transcriptomic analysis (*hns*) was confirmed to be important in surface motility. In this figure the $\Delta speB$ mutant was J15.

Evidence for a homeostatic network that maintains intracellular putrescine

The gene expression changes resulting from loss of *speB* unexpectedly suggested compensatory mechanisms for low putrescine. The transcript differences for all genes are provided in Supplemental Table 1, and for selected genes in [Table 1](#). A gene expression pattern is summarized in [Fig 11](#). The *speB* mutant had more transcripts from (a) genes of arginine and ornithine carboxylase, (b) all genes of ornithine and arginine synthesis from glutamate, and (c) genes for three separate putrescine transport systems. The *speB* mutant also had fewer transcripts from the *sap* operon which codes for a putrescine exporter ([12](#)), and from *rpmH* and *rpsT*, whose products inhibit ornithine and arginine decarboxylase ([22](#)). The net effect of these changes should be to increase intracellular putrescine: follow the solid arrows in the left half of [Fig. 11](#).

Loss of *speB* also affected pathways of energy metabolism. For instance, glutamate and α -ketoglutarate are at a branch point in metabolism which can either provide putrescine precursors or generate energy. The *speB* mutant had fewer transcripts for metabolic genes coding for all citric acid cycle enzymes, NADH dehydrogenase I (the entire *nuo* operon), cytochrome oxidase (the *cyo* operon), and several genes of menaquinone (the *menFDHBC* operon), ubiquinone, and heme synthesis. In addition to these differences, the *speB* mutant also had fewer transcripts for all genes of iron acquisition, which is consistent with diminished energy generation. In sum, this pattern suggests that low putrescine diverts metabolic flux from energy-generating pathways to putrescine synthesis.

One of the most differentially expressed gene between the parental and *speB* mutant strains was *mgtA* which had 60-fold more transcripts in the *speB* mutant and codes for the major magnesium transporter. The *speB* mutant also had more transcripts for several phosphate assimilation genes, including *phoB* which codes for the response regulator of phosphate limitation.

Discussion

PDSM and pili synthesis required putrescine synthesis and were affected by putrescine degradation and transport, as well as several transcription factors, which implies that numerous factors control intracellular putrescine concentrations. The effect of loss of putrescine transport systems on PDSM implicates extracellular putrescine on putrescine homeostasis. A requirement for the SpeA-SpeB pathway implicates extracellular arginine since SpeA is mostly periplasmic ([23](#)). The role of both putrescine synthesis and degradation suggests rapid adjustments to fluctuating putrescine concentrations. Numerous transcription factors control pili synthesis. Of these, only the gene for H-NS had fewer transcripts in the *speB* mutant, which is consistent with H-NS mediating putrescine-dependent control. This conclusion is not considered strong because of the only two-fold transcript reduction, and because of numerous changes in transcripts for other potential pili regulations in the *speB* mutant, albeit in the wrong direction. Therefore, we cannot exclude a general regulatory dysfunction mediated by depleted levels of putrescine as the basis for loss of pili synthesis.

An unexpected observation was that the *speB* mutant had more transcripts for genes whose products will maintain intracellular putrescine, i.e., compensatory mechanisms for low intracellular putrescine ([Figure 11](#)). Conversely, loss of *speB* lowered transcripts for many genes of energy metabolism and all genes of iron acquisition. This gene expression pattern suggests a diversion of metabolism away from energy generation and toward putrescine synthesis, i.e., a prioritization of putrescine synthesis over aerobic energy metabolism.

Gene (function)	log ₂ FC	FDR
Polyamine synthesis		
<i>speA</i> (putrescine)	0.99	0.003
<i>speC</i> (putrescine)	1.43	3E-4
<i>speD</i> (spermidine)	-0.83	0.01
<i>speF</i> (putrescine)	1.05	0.007
<i>rpmH</i> (inhibitor of SpeA and SpeC)	-2.77	2E-4
<i>rpsT</i> (inhibitor of SpeA and SpeC)	-1.92	8E-4
Polyamine transport		
<i>potABCD*</i> (spermidine)	-2.17	3E-4
<i>potE</i> (putrescine)	2.07	2E-4
<i>potFGHI*</i> (putrescine)	1.41	4E-4
<i>plaP</i> (putrescine)	1.11	0.005
<i>sapBCDF*</i> (putrescine export)	-1.18	8E-4
Arginine synthesis		
<i>argA_2</i> (synthesis)	1.77	0.001
<i>argB</i> (synthesis)	1.87	0.002
<i>argC</i> (synthesis)	2.86	0.005
<i>argD</i> (synthesis)	1.41	0.001
<i>argF</i> (synthesis)	1.63	0.01
<i>argG</i> (synthesis)	2.32	0.002
<i>argH</i> (synthesis)	0.97	0.001
<i>argI</i> (synthesis)	1.95	0.018
<i>argR</i> (repressor of arginine regulon)	-1.33	4E-4

Table 1.

Differentially expressed genes that are proposed to contribute to putrescine homeostasis. A positive number means more transcripts in the $\Delta speB$ (lower putrescine) strain.

Table 1. (continued)

Glutamate generation		
<i>glsA</i> (glutamine degradation)	2.16	5E-4
<i>glnG</i> (regulation of ammonia assimilation)	-1.59	0.022
TCA cycle/electron transport		
<i>acnA</i> (TCA cycle)	-1.04	8E-4
<i>acnB</i> (TCA cycle)	-1.96	2E-4
<i>fumA</i> (TCA cycle)	-1.50	6E-4
<i>fumB</i> (TCA cycle)	-1.49	0.003
<i>gltA</i> (TCA cycle)	-1.64	2E-4
<i>icdA</i> (TCA cycle)	-1.32	0.002
<i>sdhCDAB-sucABCD*</i> (TCA cycle)	-3.88	5E-5
<i>nuo</i> operon* (electron transport)	-0.82	0.004
<i>menFDHBC*</i> (electron transport)	-0.8	0.005
<i>ubiEJB*</i> (electron transport)	-0.93	0.004
<i>cyoABCD*</i> (electron transport)	-1.41	9E-4
<i>hemCD*</i> (heme)	-2.05	2E-4
Iron transport		
<i>exbBD*</i> (transport of all iron chelates)	-2.33	4E-5
<i>tonB</i> (transport of all iron chelates)	-1.9	4E-5
<i>entCEBA*</i> (enterochelin iron)	-5.2	7E-6
<i>fecABCDE*</i> (ferric citrate)	-4.4	3E-5
<i>fecIR</i> (regulators, ferric citrate)	-4.4	6E-6
<i>feoABC*</i> (ferrous iron)	-3.4	7E-4
<i>fur</i> (regulator, iron assimilation)	-0.40	0.11
Magnesium and phosphate transport		
<i>mgtA</i> (magnesium)	5.90	5E-6
<i>phoQ</i> (regulator, magnesium)	0.79	0.009
<i>pstSCAB*</i> (phosphate)	3.61	0.01
<i>phoBR*</i> (regulator, phosphate assimilation)	2.80	0.02

* Values for transcripts of the first gene of operon are given. Results for other genes of the operon are in Supplemental Table 1

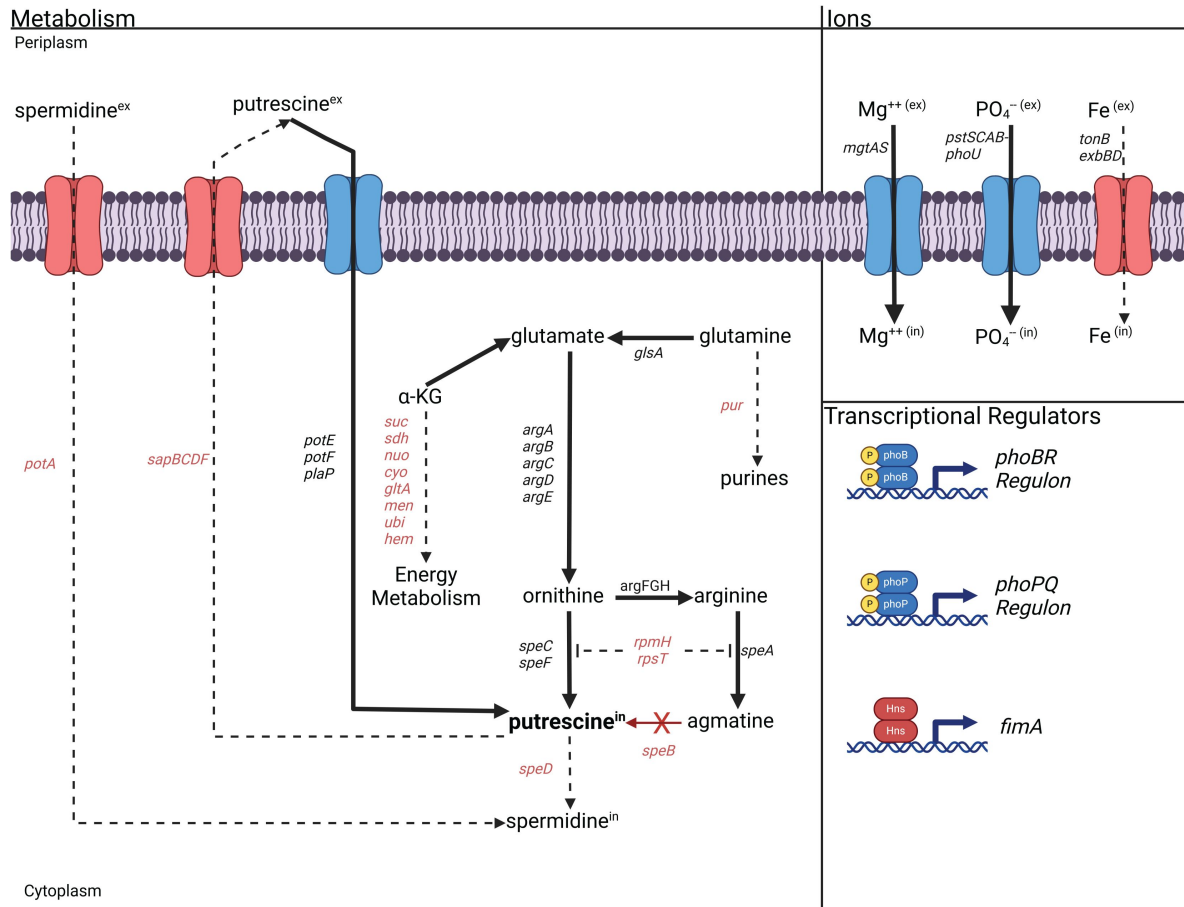


Figure 11

The deduced diversion of metabolism in a *speB* mutant away from energy metabolism toward putrescine synthesis compared to the parental strain. The effects of low putrescine are shown. Genes and processes (transport and transcriptional regulators) in red have fewer transcripts, while those in black or blue have more. The dashed lines represent proposed reduction in metabolic flux because of fewer transcripts from genes coding for the enzymes involved. Operons are shown except for the larger operons or regulons. For example, *pur* is meant to represent the unlinked genes that code for enzymes of purine synthesis. **Table 1** or Supplemental Table 1 should be consulted for specific genes and the quantitative change in transcripts.

We also propose that other changes are also compensatory mechanisms for low intracellular putrescine, specifically, more transcripts for genes coding for magnesium and phosphate transport systems in the *speB* mutant. To the extent that magnesium can directly replace putrescine, e.g., binding to nucleotides and RNA (24), magnesium could compensate for low putrescine. Our observations confirm those of J. Slauch and colleagues on an inverse correlation between intracellular magnesium and polyamines in *Salmonella* (25). They proposed an overall divalent cation homeostasis maintained by either magnesium or the polyamines (25). A rationale for the effect on phosphate metabolism is not obvious but considering the effects of low putrescine on energy metabolism, increased phosphate transport may be an adjustment to changes in energy metabolism. In any case, the substantial changes in gene expression due to low putrescine suggest multiple compensatory mechanisms, and a homeostatic gene network which because of effects on metabolic genes can rewire metabolism.

In contrast to our results, a thorough and well-designed study showed that *E. coli* PDSM requires spermidine instead of putrescine (8). The differences between the studies were strain backgrounds, media (glucose-LB vs glucose-tryptone), and incubation temperature (33° vs 37°). We found PDSM results for 37° incubations were highly variable and resulted in numerous fast-moving variants. Since growth at the higher temperature is faster, and faster growth is associated with higher putrescine, we propose that growth at 37° results in an inhibitory putrescine concentration. In this case, spermidine would be stimulatory because of its known inhibition of the putrescine biosynthetic enzymes SpeA and SpeC (26, 27) and subsequent reduction of intracellular putrescine to an optimal concentration.

The transcript changes observed in our study were different from those in the only other systematic study of putrescine's effect on *E. coli* gene expression (6). Both studies had almost identical experimental design: 1.0- or 1.14-mM putrescine was added to either a *speB* or *speB speC* mutant, respectively. Differences in strains, growth media (minimal media vs tryptone-containing media), and growth temperature (33° vs 37°) could account for the discrepancy in results. The most crucial difference could be the growth media: Igarashi and colleagues grew bacteria in a minimal medium, while we grew bacteria in an amino acid-containing medium that was required for surface motility. We propose that the slower growth in minimal medium correlates with higher guanosine tetraphosphate and in turn counters and obscures many growth-stimulatory putrescine effects. In contrast, the growth stimulation from the amino acids in our medium would not counter putrescine effects and would essentially allow detection of effects caused solely by changes in putrescine concentrations. Regardless of the explanation, common results can be interpreted as core responses to putrescine limitation. Both studies observed that low putrescine increased transcripts for genes of arginine synthesis and magnesium transport, and reduced transcripts for the *sdhCDAB-sucABCD* operon which codes for TCA cycle enzymes. The link between putrescine and arginine synthesis is probably mediated by the ArgR repressor because *argR* transcripts are 2.5-fold lower (FDR = 0.0004) in the *speB* mutant (Supplemental Table 1). Cyclic AMP may mediate putrescine-dependent control of the *sdh-suc* operon because putrescine controls translation of *cya* mRNA, which codes for adenylate cyclase, and cyclic-AMP controls the *sdhCDAB-sucABCD* operon. As noted above, the link between putrescine and magnesium transport is a recent observation (25).

Several results implicate putrescine in *E. coli* virulence during urinary tract infections. First, pili-dependent binding to the urothelium is an essential step for uropathogenic *E. coli* (28). Second, putrescine is present in urine from infected, but not healthy individuals (29, 30). Third, urinary putrescine can result from UPEC growth in urine (30) and urothelial pathophysiology (31). Putrescine's possible role in virulence is seemingly contradicted by the observation that a *speB* mutant outcompetes the parental CFT073 in the bladder of mice, i.e., less putrescine synthesis enhanced virulence (32). A majority of uropathogenic *E. coli* are associated with phylogenetic group B2—including CFT073, whereas most *E. coli* lab strains, such as W3110, are group A. Group B2 strains lack genes for the major putrescine catabolic pathway and have significantly higher

transcripts for genes coding for SpeA and SpeB (15 [↗](#)). Both observations suggest that the cytoplasmic putrescine concentration is higher in group B2 strains. We propose that the putrescine concentration in group B2 strains is normally in the inhibitory range for pili synthesis, and that loss of SpeB reduces putrescine to a level that stimulates pili synthesis. Regardless of the explanation, putrescine is likely to be important during urinary tract infections, but aspects of putrescine control of pili synthesis and other processes could be different in uropathogenic *E. coli* and is worth further examination.

Experimental Procedures

Strains

All strains used for growth rate determinations and motility assays were derivatives of *E. coli* K-12 strain W3110 and are listed in **Table 2** [↗](#). Great variations exist in standard lab strains, including W3110, from different labs. We tested nine strains, most from the Coli Genetic Stock Center, and chose our lab strain because of relative genetic stability and more quantitatively reproducible results. Our strain originated from the lab of Jon Beckwith in the 1960s via the lab of Boris Magasanik where it had been stored in a room temperature stab until the mid-1980s, at which time it was revived and frozen at -80° C. Although mutations may have accumulated during storage, its transcriptome is similar to that of other strains of the same phylogenetic group when grown in motility medium (15 [↗](#)). To construct the mutant strains, the altered allele was obtained from the Keio collection in which the gene of interest had been deleted and replaced with an antibiotic resistance gene (33 [↗](#)). The marked deletion allele was transferred into W3110 by P1 transduction (34 [↗](#)). The antibiotic gene was removed as described which generated an in-frame deletion (35 [↗](#)).

Media and Growth Conditions

For P1 transductions and plasmid transformation experiments, cells were grown in standard LB liquid medium (1% tryptone, 1% NaCl and 0.5% yeast extract, pH 7.0) at 37°C. Antibiotics were used for selection at concentrations of 25 µg/mL (both chloramphenicol and kanamycin). For growth analysis, bacteria were grown in liquid motility medium (0.5% glucose, 1% tryptone, and 0.25% NaCl) which we refer to as GT medium. Starter cultures (typically 6–12 h incubation) were grown in GT medium, harvested by centrifugation, washed twice with phosphate buffered saline and re-suspended in GT medium before inoculation. The cells were then grown at 37° C in aerobic conditions (shaking at 240 rpm) and the turbidity was measured every thirty minutes. Cell growth was measured in Klett units using a Scienceware Klett colorimeter with a KS-54 filter. 100 Klett units represent an OD₆₀₀ value of about 0.7.

Surface motility assay

For a standard surface motility assay, single colonies from fresh plates (streaked out from frozen stocks a day before) were inoculated in GT medium for six hours at 37° C in aerobic conditions (shaking at 240 rpm). 30 ml of autoclaved GT medium with 0.45% agar (Eiken, Tokyo, Japan) was poured into sterile polystyrene petri-dish (100 mm X 15 mm) and allowed to solidify at room temperature for approximately six hours. Then, 1 µL of the pre-motility growth medium was inoculated at the center of the agar plate and incubated at 33° C for 36 hrs. Each experiment was performed in triplicate and pictures were taken after 36 hours. Surface motility was extremely sensitive to humidity. Opening the incubator before 36 hours resulted in movement cessation. The surface motility assay was performed at 33° C because results from incubations at 37° C were highly variable and the cultures more frequently generated genetically stable fast-moving variants.

Table 1.

Strains

Strain Name	Genotype	Reference
BLS77	W3110 Δ <i>puuR</i> :: <i>cat</i>	(17)
BLS80	W3110 Δ <i>puuA</i> :: <i>cat</i>	(17)
BLS88	W3110 Δ <i>patA</i> Δ <i>puuA</i>	(17)
CP2	W3110 Δ <i>patA</i>	(17)
IM26	W3110 Δ <i>speB</i> :: <i>kan</i> <i>fliC-lacZ</i>	This study
IM27	W3110 Δ <i>speE</i> :: <i>kan</i> <i>fliC-lacZ</i>	This study
IM28	W3110 Δ <i>speF</i> :: <i>cat</i> <i>fliC-lacZ</i>	This study
IM29	W3110 Δ <i>speC</i> :: <i>kan</i> <i>fliC-lacZ</i>	This study
IM34	W3110 Δ <i>speC</i> Δ <i>speF</i> :: <i>cat</i> <i>fliC-lacZ</i>	This study
IM60	W3110 Δ <i>cadA</i> :: <i>kan</i> <i>fliC-lacZ</i>	This study
IM61	W3110 Δ <i>speA</i> :: <i>kan</i>	This study
IM62	W3110 Δ <i>patA</i> Δ <i>puuA</i> Δ <i>speE</i> :: <i>kan</i>	This study
IM63	W3110 Δ <i>potE</i> :: <i>kan</i>	This study
IM64	W3110 Δ <i>potF</i> :: <i>kan</i>	This study
IM65	W3110 Δ <i>plpP</i> :: <i>kan</i>	This study
IM66	W3110 Δ <i>puuP</i> :: <i>kan</i>	This study
J15	W3110 Δ <i>speB</i> Δ <i>fliC</i> :: <i>kan</i>	This study
SA1	W3110 Δ <i>fliC</i> :: <i>kan</i>	This study
SA2	W3110 Δ <i>fimA</i> :: <i>kan</i>	This study
SA3	W3110 Δ <i>fliC</i> Δ <i>fimA</i>	This study
SA4	W3110 Δ <i>hms</i> :: <i>kan</i>	This study
SA5	W3110 Δ <i>ihfA</i> :: <i>kan</i>	This study
SA6	W3110 Δ <i>lrp</i> :: <i>kan</i>	This study
W3110-LR	<i>lac</i> ⁺ <i>lacL8</i>	Lab strain

Swim assay

The media and culturing for swimming motility is identical to that for surface motility, except that the plates contained 0.25% agar and were solidified at room temperature for about one hour before inoculation. Then, 1 μ L of the culture was stabbed in the center of this swim agar plate and incubated at 33° C for 20 hours. Each experiment was performed in triplicate.

Transmission electron microscopy

Cells from surface motility plates were collected and fixed with 2.5% glutaraldehyde. Bacteria were absorbed onto Foamvar carbon-coated copper grids for 1 min. Grids were washed with distilled water and stained with 1% phosphotungstic acid for 30 s. Washed and stained grids were dried at 37°C for 10 minutes. Samples were viewed on a JEOL 1200 EX transmission electron microscope at the University of Texas Southwestern ([Figure 2](#)) and the University of Texas at Dallas ([Figure 8](#)).

ELISA analysis

Cells were grown overnight in 5 mL GT media followed by a 2-hour growth in GT media with 0.0, 0.1, 1.0, or 4.0 mM putrescine. Cells were then lysed using a 24-gauge needle. The cell lysate was diluted to a concentration of 1 mg/mL protein (based on A280) in pH 9.6 coating buffer (3 g Na_2CO_3 , 6 g NaHCO_3 , 1000 mL distilled water) and then coated onto the walls of a 96-well plate by incubating overnight at 4°C. The wells were rinsed 3 times with PBS and further blocked using coating buffer with 1% bovine albumin overnight at 4°C. The following day, wells were rinsed 3 times with PBS, and primary antibodies to FimA or RpoD were added following supplier directions. After a 2-hour room temperature incubation, secondary antibodies conjugated to HRP were added and incubated for a further two hours. HRP was activated using the TMB substrate kit (Thermo-34021) following the provided protocol. Expression was read on a BioTek plate reader based on the provided kit protocol. Data was blanked to wells only treated with bovine albumin then normalized to RpoD. Data was generated from technical and biological triplicates.

DNA isolation and genome assembly and annotation for W3110

DNA was isolated based on previous established protocols ([36](#)). Short reads were sequenced at the SeqCenter (Pittsburgh, Pennsylvania) using Illumina tagmentation-based and PCR-based DNA prep and custom IDT indices targeting inserts of 280 bp without further fragmentation or size selection steps. The Illumina NovaSeq X Plus sequencer was ran producing 2X151 paired-end reads.

Demultiplexing, quality control, and adapter trimmer was performed with bcl-convert (v4.2.4). Total Illumina Reads (R1+R2): 4059078 with 553087045 bps >Q30.

Long reads were prepared using the PCR-free Oxford Nanopore Technologies ligation sequencing kit with the NEBNext companion module. No fragmentation or size selection was performed. Long read libraries were performed using R10.4.1 flow cell. The 400bps sequencing mode with a minimum read length of 200bp were selected. Guppy (v6.5.7) was used at the super-accurate basecalling, demultiplexing, and adapter removal. Total long reads: 53773 with 89.607 % of bps > Q20.

To generate the completed genome, porechop (v0.2.4) was used to trim residual adapter sequences from long reads. Flye (v2.9.2) was used to generate the *de novo* assembly under the nano-hq model. Reads longer than the estimated N_{50} based on a genome size of 6Mbp initiated the assembly. Subsequent polishing using the short read data was performed using Pilon (v1.24) under default parameters. Long read contigs with an average short read coverage of 15X or less were removed from the assembly. The assembled contig was confirmed to be circular via circulator

(v1.5.5). Annotation was performed using prokka (v 1.14.6). Finally, statistics were recorded using QUAST (v5.2.0). The final genome contained 1 contig of 4750347 bp with a sequencing depth of 123.92x. The N_{50} was 4750347. This genome can be accessed via the accession number CP165600 or via the BioProject PRJNA1142534 via NCBI.

The completed genome has an estimated average nucleotide identity of 99.9694 to the W3110 genome deposited to NCBI (genome assembly ASM1024v1).

RNA isolation and quality control

Cells were grown for 2 hours in 1 mL GT media. 60 μ L were then added to 1 mL of fresh GT, with or without 1 mM putrescine, and grown for another two hours. After growth, the cells were centrifuged, and frozen at -80°C . The isolation and analysis protocol has been previously published (15 [↗](#)). Cell pellets were thawed, resuspended in 0.7 mL of buffer RLT (Qiagen RNeasy kit), and mechanically lysed using a bead beater (FastPrep-24 Classic from MP Biomedical) set at the highest setting for three 45-s cycles with a 5-min rest period on ice between cycles. Cell lysates were used to isolate RNA using a Qiagen RNeasy mini kit. RNA was quantified, DNase treated, and re-quantified on a nanodrop. RNA that passed the first quality check was analyzed on RNA-IQ (Qubit). RNA with an IQ higher than 7.5 was used. RNA that passed both checks were run on an agarose gel to check for RNA integrity. RT-qPCR was used to ensure there was no DNA contamination. RNA libraries that passed all these quality checks were submitted to the genomics core facility at the University of Texas at Dallas for RNA sequencing. The core performed rRNA removal (RiboMinus Transcriptome Isolation Kit or RiboCop bacterial rRNA depletion—Lexogen), library preparation (Stranded Total RNA Prep—Illumina), and single-end Illumina sequencing.

Reverse transcription quantitative PCR

RNA was isolated as described above. One microgram of RNA was reverse transcribed using LunaScript RT Super Mix (NEB E3010) and another microgram was subjected to the same reaction as the reverse transcribed RNA, except without reverse transcriptase (negative control) following manufacturer instructions. The cDNA was then diluted to 10 ng/ μ L for qPCR. PCR reactions were composed of 10 ng cDNA, 8 μ L of nuclease free water, 10 nM primers (*rpoD*: Forward: TCGTGTGAAGCAGAAGAAGCG; Reverse: TCGTCATCGCCATCTTCTTCG) (*fimA*: Forward: ATGGTGGGACCGTTCACTTT; Reverse: GGCAACAGCGGCTTTAGATG), and PowerUp SYBR Green 2X master mix for qPCR (ThermoFisher A25777) in a 20 μ L reaction. A Quantstudio 3 qPCR machine was used to generate critical threshold (C_t) values. The C_t values were then analyzed using the $2^{-\Delta\Delta C_T}$ method (37 [↗](#)) using *rpoD* as a library control.

RNAseq Analysis

Transcripts were aligned to the W3110 genome and CDS (CP165600) available on NCBI using CLC genomics workbench (Qiagen version 22.00) for RNA-seq analysis. The gene expression counts generated as this output were used to perform differentially expressed gene (DEG) analysis using EdgeR (version 3.38.4) to analyze read counts (38 [↗](#), 39 [↗](#)). DEGs had an adjusted P-value less than 0.05.

Data availability

The raw RNA seq reads were deposited in GenBank: BioProject accession number PRJNA1126736. The genome was deposited in GenBank: BioProject accession number PRJNA1142534. The genome accession number is CP165600.

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Reviewer #1 (Public review):**Summary:**

Ita Mehta and colleagues have investigated the role of putrescine in the pili-dependent surface motility of a laboratory strain of *Escherichia coli*. Enterobacteria, and particularly *E. coli* and *Salmonella Typhimurium* contain an enormous amount of putrescine and cadaverine compared to other bacteria. It has been estimated by Igarashi and colleagues that putrescine is present in *E. coli* at levels of at least 30 mM. Therefore, an investigation of the role of putrescine in *E. coli* is a welcome and important contribution to understanding polyamine function. The authors have used a comprehensive suite of *E. coli* gene deletion strains of putrescine biosynthetic, transport, and catabolic genes to understand the role of putrescine in pili-dependent surface motility.

Strengths:

Single gene deletions of arginine decarboxylase (*speA*) and agmatine ureohydrolase (*speB*), and a double gene deletion of the constitutive ornithine decarboxylase (*speC*) and the acid-inducible ornithine decarboxylase (*speF*), all of which are involved in putrescine biosynthesis, were found by the authors to be less efficient at pili-dependent surface motility. In addition, the putrescine transport genes *plaP* and *potF* are also required for efficient pili-dependent surface motility. Furthermore, the putrescine catabolic genes *patA* and *puuA*, when co-deleted, reduce pili-dependent surface motility. Transcriptomic analysis of the agmatine ureohydrolase (*speB*) gene deletion strain compared to the parental strain indicates a coordinated response to the *speB* gene deletion, including upregulation of ornithine biosynthetic genes and a downregulation of energy metabolic genes.

Weaknesses:

Because the cellular content of putrescine and other polyamines in the *E. coli* strains was not measured at any point in this study, and the gene deletions were not genetically

complemented, it is not possible to definitively attribute physiological changes to the gene deletion strains specifically to changes in putrescine levels. Furthermore, the GT medium used for the mobility experiments contains trypsinated casein (tryptone), which may contain polyamines and most certainly contain arginine. There are two modes of putrescine biosynthesis in *E. coli*: one mode is the direct formation of putrescine from L-ornithine mediated by ornithine decarboxylase, and the other is the indirect pathway involving decarboxylation of arginine to form agmatine, followed by hydrolysis of agmatine to form putrescine and urea. In the absence of external arginine, putrescine is made by ornithine decarboxylase, however, in the presence of external arginine, ornithine biosynthesis is repressed and arginine decarboxylase becomes the primary biosynthetic route for putrescine biosynthesis. The GT medium used by the authors will tend to favor putrescine production from arginine. The *speB* gene deletion, which is used for the transcriptomic analyses, will even in the absence of external arginine, accumulate a very large amount of agmatine, greater than the level of putrescine. This will confound the interpretation of the effect of the *speB* gene deletion, because agmatine accumulation may be responsible for some of the effects, and the addition of external putrescine may repress agmatine accumulation. In the absence of polyamine level measurements, the relative levels of agmatine, the putrescine structural analog cadaverine, and the accumulation of decarboxylated S-adenosylmethionine, are not known. Changes to these metabolites could affect pili-dependent surface motility. Furthermore, it is not possible to conclude that the effects of gene deletions to biosynthetic, transport or catabolic genes on pili-dependent surface motility are due to changes in putrescine levels unless one takes it on faith that there must be changes to putrescine levels. Since *E. coli* contains such an enormous amount of putrescine, it is important to know how much putrescine must be depleted in order to exert a physiological effect.

The authors have tackled an important biomedical problem relevant to infections of the urogenital tract and also important for understanding the very unusual high level of putrescine in *E. coli* and related species. However, without confirmation of putrescine levels in their various strains, it would be difficult to unequivocally conclude that putrescine, or changes to its concentrations, are responsible for the physiological changes seen with the gene deletion strains.

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Reviewer #2 (Public review):

Summary:

Mehta et al., in constructing *E. coli* strains unable to synthesize polyamines, noted that strains deficient in putrescine synthesis showed decreased movement on semisolid agar. They show that strains incapable of synthesizing putrescine have decreased expression of Type I pilin and, hence, decreased ability to perform pilin-dependent surface motility.

Strengths:

The authors characterize the specific polyamine pathways that are important for this phenomenon. RNAseq provides a detailed overview of gene expression in the strain lacking putrescine. The data suggest homeostatic control of polyamine synthesis and metabolic changes in response to putrescine.

Weaknesses:

In this version, the authors ignore phase variation of the pil operon promoter, which can be monitored via PCR. The gene expression data suggest that shifting to the pilin "off" state could help explain the phenotype.

Reviewer #3 (Public review):

Summary:

This study by Mehta et al. describes the mechanisms behind the observation that putrescine biosynthesis mutants in *Escherichia coli* strain W3110 are affected by surface motility. The manuscript shows that the surface motility phenotype is dependent on Type I fimbriae and that putrescine levels affect the expression level of fimbriae. The results further suggest that without putrescine, the metabolism of the cell is shifted towards the production of putrescine and away from energy metabolism.

There are two main aspects in the manuscript.

(1) The first observation is that a *fimA* mutant modified/decreased the motility phenotype. From this result, the authors conclude that type I fimbriae (or pili) are involved in the surface motility phenotype. Type I fimbriae are typically known to be involved in non-motile phenotypes, such as biofilm formation or adhesion. Type I fimbriae are also co-regulated with other surface structures that might impact motility. Thus, more controls are needed before concluding that the surface motility requires the type I fimbriae. For instance, the authors should have complemented the mutants and should have verified the flagella expression/motility in the *fimA* mutant.

(2) The second observation is that putrescine also impacts the surface motility phenotype and the expression of type I fimbriae. Although there is no genetic complementation, here the exogenous addition of putrescine to the *speB* mutant provides a chemical complementation method, which makes the data stronger.

In addition, testing the effect of putrescine on motility and type I fimbriae expression in additional strains of *E. coli* would strengthen the conclusion. This is especially important since the results are somewhat different from previous results obtained with a different strain of *E. coli*. The authors do note that experimental conditions are different, but testing their theory would make the conclusions stronger.

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