ORIGINAL RESEARCH

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Hysteresis in *myo*-inositol utilization by *Salmonella* Typhimurium

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Abstract

Growth of *Salmonella enterica* serovar Typhimurium strain 14028 with *myo*-inositol (MI) as the sole carbon and energy source is characterized by a bistable phenotype that manifests in a growth phenotype with an extraordinarily long and length-variable lag phase. However, in the presence of hydrogen carbonate, in the absence of IolR that represses the MI degradation pathway, or if cells are already adapted to minimal medium (MM) with MI, the lag phase is drastically shortened, and the bistable phenotype is abolished. We hypothesized that memory development or hysteresis is a further characteristic of MI degradation by *S.* Typhimurium; therefore, we investigated the transition from a short to a long lag phase in more detail. Growth experiments demonstrated that memory on the population level is successively lost within approximately 8 hr after cells, which had been adapted to MI utilization, were transferred to lysogeny broth (LB) medium. Flow cytometry (FC) analysis using a chromosomal fusion to P_{iolE}, a promoter controlling the expression of the enzymatic genes *iolE* and *iolG* involved in MI degradation, indicated a gradual reversion within a few hours from a population in the "ON" status with respect to *iolE* transcription to one that is mainly in the "OFF" status. Growth and FC experiments revealed that IolR does not affect hysteresis.

KEYWORDS
hysteresis, metabolism, *myo*-inositol, *Salmonella*

1 | INTRODUCTION

Phenotypic variation is widespread among prokaryotes. Its molecular mechanisms include genetic changes such as genomic inversion and strand-slippage mechanisms, epigenetic variations dependent on DNA methylation, and feedback-based bi- or multistability characterized by at least two distinct phenotypes within an isogenic population (Smits, Kuipers, & Veening, 2006). This phenomenon was described for the first time for lactose utilization by *Escherichia coli* requiring the expression of the *lac* operon that is negatively regulated by LacI.

Phenotypic heterogeneity that affects the fitness of pathogenic bacteria is of particular interest. For example, the survival of *Staphylococcus aureus* against antibiotic treatment requires that some cells, called "persisters", enter a condition of reduced growth (Balaban, Merrin, Chait,

Kowalik, & Leibler, 2004). Further evidence for a correlation between heterogeneity and virulence properties comes from recent observations with *S. enterica* serovar Typhimurium (*S.* Typhimurium), a major cause of food poisoning worldwide. *S.* Typhimurium infects both animal and human hosts and causes enteric fever, gastroenteritis, bacteremia, and systemic infection. In mice, it evokes a disseminated infection that serves as a model for human typhoid fever. When this pathogen infects the host, a stochastic switch takes place that results in an invasive and therefore self-destructive fraction, and a noninvasive subpopulation that benefits from the dying one, possibly by alleviating competition by commensals (Ackermann et al., 2008). More recently, it was observed that within heterogeneous populations, nondividing *Salmonella* cells and those that express virulence factors survived best after exposure to antibiotics (Arnoldini et al., 2014; Claudi et al., 2014).

Growth of _S._ Typhimurium with _myo_-inositol (MI) as the sole carbon and energy source also exhibits a bistable phenotype (Kröger, Srikumar, Ellwart, & Fuchs, 2011) . The _iol_ genes of _S._ Typhimurium 14028 located on the genomic island GEI4417/4436 are responsible for MI degradation, which involves five enzymes encoded by this island, resulting in the formation of dihydroxyacetone phosphate, acetyl coenzyme A, and CO_2 (Kröger & Fuchs, 2009). The phenotypic heterogeneity correlates with the bistable expression of the promoter P$_{iolE}$, which controls the production of IolE and IolG that catalyze the first steps in MI degradation. The regulator IolR represses all but one promoter of the _iol_ divergon, including that of its own gene and of _iolT1_ encoding the predominant MI transporter (Kröger, Stolz, & Fuchs, 2010). An intermediate of MI degradation, 2-deoxy-5-keto-D-gluconic acid 6-phosphate (DKGP), antagonizes IolR binding, thus inducing the expression of _iol_ genes (Yoshida, Shibayama, Aoyama, & Fujita, 1999; Yoshida et al., 2008). The _Salmonella_-specific activator ReiD induces the transcription of _iolE_, which is not controlled by IolR, and is assumed to trigger a metabolic response during infection (Rothhardt, Kröger, Broadley, & Fuchs, 2014). A striking feature of _S._ Typhimurium 14028 is its long lag phase in the presence of MI with a high variability under the same experimental conditions. However, length and variability are abolished in the absence of the _iol_ gene repressor IolR or by the presence of at least 0.55% CO_2 (Kröger et al., 2011). Recently, using fluorescence microscopy and flow cytometry (FC) analysis, we demonstrated that at the single-cell level, the pronounced heterogeneity of an _S._ Typhimurium population during nonadapted growth on solid minimal medium (MM) with MI is correlated with the bistable behavior of at least one _iol_ gene promoter, P$_{iolE}$, and that only a small subpopulation exhibits an induced _iolE_ promoter (Kröger et al., 2011).

A common characteristic of bistable systems is hysteresis, defined as a situation in which the transition from one state to another requires a force unequal to that required for the reverse transition (Smits et al., 2006; Veening, Smits, & Kuipers, 2008), or less stringently, a situation within a biological system in which the state is not solely determined

by the present conditions, but also depends on its history (Casadesus & D'Ari, 2002; Wolf et al., 2008). In this study, we analyzed quantitative growth and FC data of _S._ Typhimurium and its mutants following a shift from lysogeny broth (LB) medium to MM with MI and _vice versa_. We observed hysteresis that allows _S._ Typhimurium to rapidly restart growth with MI during a memory phase of approximately 8 hr.

2 | RESULTS AND DISCUSSION

2.1 | Hysteresis in MI utilization

In comparison with cells pre-grown in a rich medium, the lag phase of an _S._ Typhimurium culture adapted to growth with the polyol MI as the sole carbon and energy source is much shorter (Kröger & Fuchs, 2009). Under this experimental condition, the bistable phenotype is abolished as reflected by a homogeneous growth behavior and low variability in the duration of the lag phase, resembling that in rich medium. This preliminary observation prompted us to investigate whether or not a memory effect or hysteresis can be observed during the growth of _S._ Typhimurium with MI. Strain 14028 was streaked out on MI agar plates and cultivated for 2 days. Several colonies were resuspended in MM with MI, and the optical density at 600 nm (OD$_{600}$) was adjusted to 0.8. Aliquots were then diluted 1:500 in LB medium and cultivated without shaking for 4, 6, and 8 hr at 37°C. Aliquots of these LB cultures with 2×10^5 cells were then used to inoculate MM with MI. To assure that each inoculum contained an equal amount of cells, the cfu/ml were determined in preliminary experiments and controlled by plating. Following monitoring of the bacterial growth, we identified the lag phase in MM/MI to end 23, 36, and 44 hr after the incubation of 4, 6, and 8 hr, respectively, in LB medium, in comparison with a lag phase of 20 hr in a control culture whose inoculum had no contact with rich medium (Figure 1). By plating aliquots of cultures in the lag phase, we calculated a division rate in MM/MI of 0.1 hr^{-1} with cells from rich medium, and of 0.3 hr^{-1} with cells preadapted to MI, corresponding well
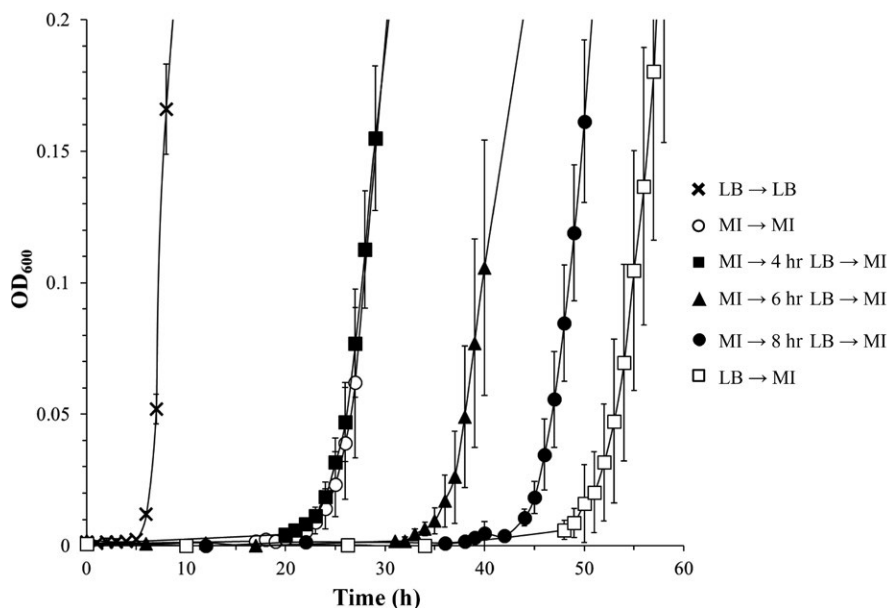

FIGURE 1 Hysteresis of _myo_-inositol (MI)-adapted _S._ Typhimurium. Strain 14028 was pre-grown in minimal medium (MM) with MI, diluted 1:500 into lysogeny broth (LB) medium, and incubated for 4, 6, and 8 hr. An equal number of cells were then transferred into fresh MM with MI. MM is M9 medium supplemented with 2 mmol/L MgSO$_4$, 0.1 mmol/L CaCl$_2$ supplemented with 55.5 mmol/L (1%, wt/vol) MI. Bacterial growth curves were obtained from cultures incubated at 37°C without agitation in 250 ml bulb flasks with 50 ml medium. OD$_{600}$ was measured at the indicated time intervals. Standard deviations were calculated from triplicates

to the lengths of the lag phases shown in Figure 1. These data suggest that MI-adapted cells maintain the capability to utilize this substrate even when they are surrounded by a rich medium for several hours. This memory effect or hysteresis gradually decreases with increasing time of incubation in the medium free of MI and is nearly completely abolished after the cells had been in contact with LB medium for at least 8 hr. Thus, the memory effect allows _S._ Typhimurium to reshift its metabolism to MI utilization, at least during a period of several hours, in case that the better carbon source is not further available.

2.2 | Determination of hysteresis on a single-cell level

To investigate the behavior of a _S._ Typhimurium cell culture upon medium switch, we first performed FC analysis of strain MvP101 P$_{iolE}$::_gfp_ (Table 1), which carries a chromosomal fusion of the _iolE_ promoter (P$_{iolE}$) with _gfp_, during lag phase in MM/MI in correlation with its division rates. We observed an increase in fluorescence intensity by a factor of 37 within 22 hr, whereas less than six cell divisions were calculated during a 36-hr lag phase (Table S2). Thus, the increase in fluorescence activity during the lag phase rather reflects the switch of OFF cells to ON cells than the division of cells that are already in the ON status. We therefore conclude that the cell population in the lag phase in MM/MI harbors a subpopulation of cells that change their metabolism to MI utilization.

According to the observed hysteresis, we assumed that _S._ Typhimurium continuously downregulates the _iol_ genes after MI was depleted from the medium. To test this hypothesis, we adapted strain MvP101 P$_{iolE}$::_gfp_ to MI by several passages on MI agar plates. An aliquot of an overnight culture in MI medium was diluted 1:500 in LB medium. The growth behavior of this culture was monitored, and samples were taken each hour over a period of 12 hr, and after 24 hr to monitor

the transcriptional response of P$_{iolE}$ by FC measurements. This single-cell analysis revealed a rapid decrease in the fluorescence intensity of GFP within a few hours of incubation in LB medium (Figure 2; Table 2). To exclude that the steady decrease of the P$_{iolE}$ activity is caused by a reduced metabolism during adaptation of _S._ Typhimurium to LB medium, and/or by GFP degradation and dilution, a control strain with a _gfp_-fusion to the promoter of the housekeeping gene _rpsM_ was investigated in the same manner. This gene encodes the ribosomal protein S13 and is described previously for constitutive expression in _E. coli_ (Nikolic, Barner, & Ackermann, 2013). More than half of the population of MvP101 P$_{rpsM}$::_gfp_ cells exhibited fluorescence directly after inoculation in LB. The amount of cells in the ON status with respect to _rpsM_ transcription increased to 80% after 1 hr, and to more than 95% after 5 hr (Table 2). These data suggest that when cells are reinoculated in LB medium, they rapidly shift their metabolism from MI to other carbon sources, and an increasing percentage of cells switches down the transcriptional activity of P$_{iolE}$ upon contact with LB medium.

We then correlated these FC data with the division rates of _S._ Typhimurium during 8 hr of growth in LB (Table S2). Within the first 2 hr in the absence of MI, the cell numbers and the fluorescence intensity increased, suggesting a proliferation of ON cells. Afterwards, a strong increase in the cell density was observed during exponential phase, whereas the fluorescence intensity and thus the amount of ON cells decreased, indicating that the population of ON cells switches to the OFF status or does not further divide. After 8 hr, the samples still exhibit a low level of fluorescence, an observation that is in line with a weak memory of this cell population in comparison with an overnight LB culture.

2.3 | Hysteresis is independent of repressor _IolR_

The single-cell analysis correlated well with the _S._ Typhimurium growth curves of Figure 1, suggesting that the amount of cells harboring an

TABLE 1 Strains and plasmids used in this study

	Description and relevant features	Source or literature
Bacterial strains		
14028	_S. enterica_ serovar _Typhimurium_ strain ATCC14028	ATCC
14028 Δ_iolR_	In-frame _iolR_ (STM4417) deletion mutant	This study
MvP101	14028 with _sseD_::_aphT_, Kan^R; allelic-exchange mutant	(Medina et al., 1999)
MvP101 Δ_iolR_	In-frame _iolR_ (STM4417) deletion mutant of MvP101	This study
Plasmids		
pKD3	_pir_-dependent, FRT sites, Cm^R	(Datsenko & Wanner, 2000)
pKD4	_pir_-dependent, FRT sites, Kan^R	(Datsenko & Wanner, 2000)
pKD46	Lambda-Red helfer plasmid; Amp^R	(Datsenko & Wanner, 2000)
pCP20	FLP recombinase plasmid; Amp^R	(Datsenko & Wanner, 2000)
pUTs-_gfp_(Cm^R)	Replacement of _lux_ with _gfp_ from pPROBE-NT in a transposase-negative derivate of pUT mini-Tn5 luxCDABE Km2; suicide plasmid, _mob_RP4, _ori_ R6K, _gfp_, Cm^R	(Starke, Richter, & Fuchs, 2013)
pUTs-P$_{iolE}$::_gfp_	pUTs-_gfp_(Cm^R) with 500 bp putative promoter region of _iolE_ (STM4424) cloned in front of _gfp_	This study
pUTs-P$_{rpsM}$::_gfp_	pUTs-_gfp_(Cm^R) with 500 bp putative promoter region of _rpsM_ (STM3418) cloned in front of _gfp_	This study

TABLE 2 FC data analysis for % values of ON cells (>3*10[2] FI) of MvP101 P$_{iolE}$::_gfp_ and P$_{rpsM}$::_gfp_ during hysteresis and in MI medium as a control

	MvP101 P$_{iolE}$::_gfp_		MvP101 P$_{rpsM}$::_gfp_	
time [hr]	LB	MM + MI	LB	MM + MI
0	43.6	27.3	69.3	29.1
1	57.9	–	80.6	–
2	58.3	–	84.9	–
3	40.1	–	80.1	–
4	2.7	37.5	79.4	66.5
5	0.7	–	95.0	–
6	0.3	77.2	97.7	98.1
7	0.3	79.4	98.7	95.2
8	0.3	91.2	98.6	98.0
9	0.1	–	–	–
10	0.1	–	–	–
11	0.1	–	–	–
12	0.1	–	–	–
24	0.1	77.8	–	74.1

FC, Flow cytometry; LB, lysogeny broth; MI, _myo_-inositol; MM, minimal medium.
Data are an average of independent measurements. –, not measured

induced _iolE_ promoter contributes to hysteresis. To test this assumption, we repeated the experiments described above with mutant 14028 Δ_iolR_, which lacks the repressor IolR and exhibits a constitutive expression of all _iol_ genes (Kröger & Fuchs, 2009). In comparison with parental strain 14028, the lag phases of the mutant were shorter due to a lack of

IolR as expected (Figure 3a), and the standard variations of most data points were significantly lower, confirming that IolR reduces the high variability observed for the growth of _S._ Typhimurium with MI (Kröger & Fuchs, 2009). Surprisingly, the elongation of the lag phase in MM/MI again positively correlated with the incubation time in LB medium, thus resembling the pattern previously observed (Figure 1). As a control, we performed FC with strain MvP101 Δ_iolR_ P$_{iolE}$::_gfp_ as described above. Within the first 3 hr following the depletion of MI, a reduction in the fluorescence activity was observed (Figure 3b). When the culture subsequently reached the end of the lag phase and entered the exponential phase, the amount of GFP increased, reflecting the derepression of the _iolE_ promoter in the absence of IolR. These data suggest that loss of memory for MI utilization even takes place when the _iol_ genes are not repressed. Taken together, we conclude that the phenomenon of hysteresis during growth of _S._ Typhimurium with MI is independent of the repressor IolR and probably also of the IolR-antagonist DKGP.

3 | CONCLUSION

Examples for the experimental demonstration of hysteresis in bacteria are rare. Recently, it was demonstrated with _Caulobacter crescentus_ that exposure to a moderate concentration of sodium chloride contributed to the survival at higher concentrations, but in dependence of the time interval between the exposures (Mathis & Ackermann, 2016). Two types of nongenetic memory, the phenotypic and the response memory, revealed to play a role for _E. coli_ in the adaptation to fluctuating carbon sources {Lambert, 2014 #777}, and the motile state of _Bacillus subtilis_ was found to be memoryless in contrast to the sessile state {Norman, 2013 #776}. In _S._ Typhimurium the transcriptional heat-shock response
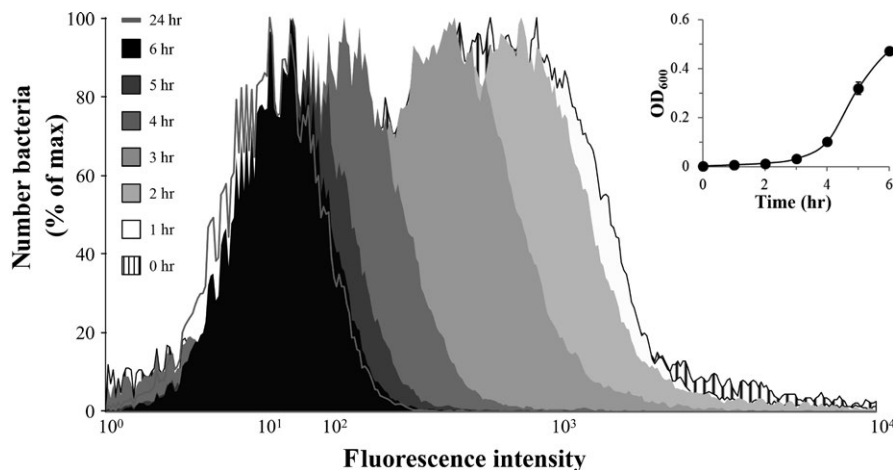

FIGURE 2 Flow cytometry (FC) analysis of MvP101 P$_{iolE}$::_gfp_. The reporter strain was pre-grown for several passages in minimal medium (MM) with _myo_-inositol (MI) and diluted 1:500 into 50 ml of fresh lysogeny broth (LB) medium. During growth, the OD$_{600}$ was measured at the indicated time points, and samples were diluted in 1% phosphate-buffered saline containing 2% formaldehyde to stabilize GFP (Bongaerts, Hautefort, Sidebotham, & Hinton, 2002). Fluorescence between 515 nm and 545 nm was measured with a FACS Aria I flow cytometer from Becton Dickinson at 488 nm excitation wavelength. A total of 10,000 events were monitored, and the collected data were analyzed using Flowing Software (v 2.5.1). The abscissa indicates the green fluorescence intensity in bi-exponential scale, and the ordinate the number of bacteria in relation to the maximal cell counts. Each histogram represents the mean of three independent measurements. The insert illustrates the growth phase of the population during sample acquisition; the standard deviation was calculated from three triplicates. For all FC experiments, bacterial strains were grown overnight at 37°C in LB medium (Sambrook & Russell, 2001) or in MM with MI
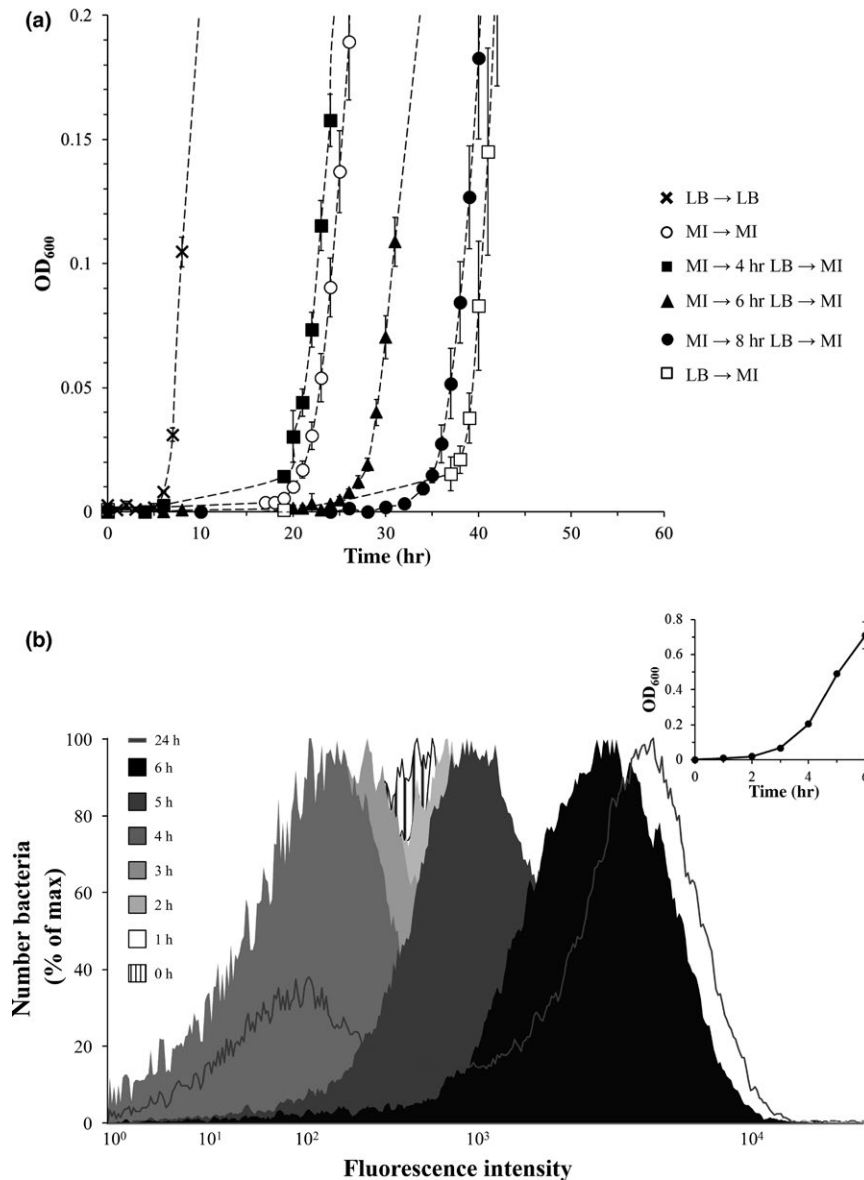
FIGURE 3 Hysteresis of *myo*-inositol (MI)-adapted *S.* Typhimurium 14028 cells is IolR-independent. (a) Growth curves of strain MvP101 Δ*iolR*. The in-frame *iolR* (STM4417) deletion mutant of strain MvP101 was constructed by the one-step method based on the phage λ Red recombinase (Datsenko & Wanner, 2000; Kröger & Fuchs, 2009); see Table 1 for plasmids and Table S1 for oligonucleotides. To obtain phage-free colonies, bacteria were cultivated on green indicator plates (Provence & Curtiss, 1994). Experimental conditions were identical to those described in the legend of Figure 1. Growth curves are shown by dashed lines. (b) Flow cytometry (FC) analysis of MvP101 Δ*iolR* P*iolE*::*gfp*. The fluorescence activity of P*iolE* was monitored in the strain lacking *iolR*. The experiment was performed as described in the legend of Figure 2

is characterized by such a memory effect, as a majority of heat shock-induced genes remain upregulated when the stress condition has already ceased (Pin et al., 2012). Here, we show that hysteresis is also a feature of *S.* Typhimurium when it encounters a change from MM with MI to one with a more efficient energy source. The absence of MI results in a gradual loss of hysteresis within a few hours, suggesting *S.* Typhimurium has a low preference for MI, which is present only in a few of the metabolic niches of this pathogen such as the gut or soil with decaying plant matter.

At a first glance, our results suggest that the gradual transition of the genes for MI degradation from induction to silencing constitutes the loss of memory. Surprisingly, the locking of cells into the ON status of *iol* gene transcription does not abolish hysteresis as demonstrated

here using a IolR-negative mutant. We therefore conclude that IolR is not responsible for the hysteresis observed here, and further investigations are required to identify the factors determining the memory of a passed metabolic condition.

CONFLICT OF INTEREST

None declared.

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