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## ***msbB* deletion confers acute sensitivity to CO<sub>2</sub> in *Salmonella enterica* serovar Typhimurium that can be suppressed by a loss-of-function mutation in *zwf***

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### Abstract

**Background:** Pathogens tolerate stress conditions that include low pH, oxidative stress, high salt and high temperature in order to survive inside and outside their hosts. Lipopolysaccharide (LPS), which forms the outer-leaflet of the outer membrane in Gram-negative bacteria, acts as a permeability barrier. The lipid A moiety of LPS anchors it to the outer membrane bilayer. The *MsbB* enzyme myristoylates the lipid A precursor and loss of this enzyme, in *Salmonella*, is correlated with reduced virulence and severe growth defects that can both be compensated with extragenic suppressor mutations.

**Results:** We report here that *msbB* (or *msbB somA*) *Salmonella* are highly sensitive to physiological CO<sub>2</sub> (5%), resulting in a 3-log reduction in plating efficiency. Under these conditions, *msbB Salmonella* form long filaments, bulge and lyse. These bacteria are also sensitive to acidic pH and high osmolarity. Although CO<sub>2</sub> acidifies LB broth media, buffering LB to pH 7.5 did not restore growth of *msbB* mutants in CO<sub>2</sub>, indicating that the CO<sub>2</sub>-induced growth defects are not due to the effect of CO<sub>2</sub> on the pH of the media. A transposon insertion in the glucose metabolism gene *zwf* compensates for the CO<sub>2</sub> sensitivity of *msbB Salmonella*. The *msbB zwf* mutants grow on agar, or in broth, in the presence of 5% CO<sub>2</sub>. In addition, *msbB zwf* strains show improved growth in low pH or high osmolarity media compared to the single *msbB* mutant.

**Conclusion:** These results demonstrate that *msbB* confers acute sensitivity to CO<sub>2</sub>, acidic pH, and high osmolarity. Disruption of *zwf* in *msbB* mutants restores growth in 5% CO<sub>2</sub> and results in improved growth in acidic media or in media with high osmolarity. These results add to a growing list of phenotypes caused by *msbB* and mutations that suppress specific growth defects.

## Background

Lipopolysaccharide (LPS), the most abundant molecule on the surface of Gram-negative bacteria, acts as a permeability barrier and renders the outer-leaflet of the outer membrane (OM) relatively impermeable to hydrophobic antibiotics, detergents [1], and host complement [2]. LPS consists of three major components: lipid A, core polysaccharides and O-linked polysaccharides. Lipid A, with its fatty acid anchors [lauric, myristic and sometimes palmitic acid], is an endotoxin primarily responsible for TNF $\alpha$ -mediated septic shock. The addition of myristic acid to the lipid A precursor is catalyzed by the enzyme MsbB [3].

It has been shown that *msbB* *Salmonella* serovar Typhimurium exhibits severe growth defects in LB and sensitivity to bile salts (MacConkey) and EGTA-containing media. However, compensatory suppressor mutants can be isolated that grow under these conditions. One of these suppressor phenotypes results from a mutation in *somA*, a gene of unknown function [4]. *msbB* *Salmonella* Typhimurium strains have recently been developed as potential anti-cancer agents that possess impressive anti-tumor activity in mice [5]. In a phase I clinical study *msbB* *Salmonella* were shown to be safe in humans when administered *i.v.* However, bacteria were rapidly cleared from the peripheral blood of humans and targeting to human tumors was only observed in few patients at the highest dose levels of  $3 \times 10^8$  CFU/m<sup>2</sup> and  $1 \times 10^9$ /m<sup>2</sup> [6]. Toso et al. [6] noted that YS1646 (suppressed *msbB* strain, see below) grew best in air without added CO<sub>2</sub>.

The potential to grow in acidic and CO<sub>2</sub>-rich environments is a hallmark of pathogenic bacteria, enhancing persistence within phagocytes and survival inside the host. Sensitivity to CO<sub>2</sub> and low pH of *msbB* *Salmonella* strains might explain poor colonization of tumors, which often contain high levels of CO<sub>2</sub> and lactic acid [7,8] due to the Warburg effect, also known as aerobic glycolysis, whereby glucose uptake is elevated while oxidative phosphorylation is reduced, even in the presence of oxygen. Our previous work on suppressors of *msbB* *Salmonella* raised the possibility that secondary mutations could suppress sensitivity to 5% CO<sub>2</sub> and acidic conditions.

Here we report that the growth of *msbB* *Salmonella* is highly inhibited (greater than 3-log reduction in plating efficiency) in a 5% CO<sub>2</sub> atmosphere in LB media as well as under low pH conditions when compared to wild-type *Salmonella*. Furthermore, several CO<sub>2</sub> resistant clones were selected from an *msbB* *Salmonella* transposon library (Tn5). Three mutations were mapped and all were shown to contain the Tn5 marker in the *zwf* gene, which encodes

the enzyme glucose-6-phosphate-dehydrogenase and is tightly linked to the *msbB* gene.

## Results

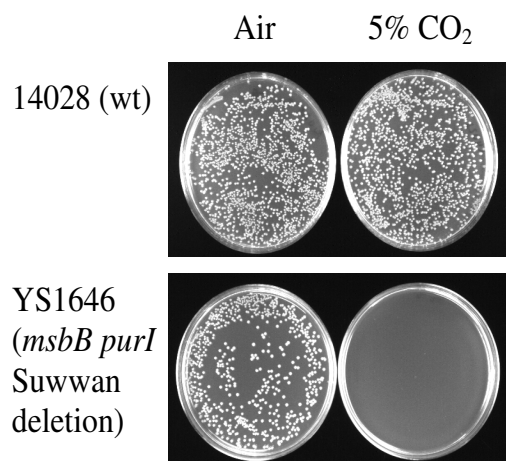
### CO<sub>2</sub> sensitivity of *msbB* *Salmonella*

CO<sub>2</sub> sensitivity was first observed when YS1646, an *msbB purI* Suwwan deletion strain of *Salmonella* Typhimurium, was plated on blood or LB plates and incubated in a 5% CO<sub>2</sub> incubator (Caroline Clairmont, personal communication; Toso et al., 2002). Suwwan deletion strains lack ~100 genes in the 17.7 to 19.9 Cs region of the chromosome [9]. In our studies, plating identical amounts (e.g., 100  $\mu$ l of a 10<sup>-5</sup> dilution of a culture grown under non-selective conditions) to duplicate plates incubated at 37°C in either air or 5% CO<sub>2</sub>, few or no colonies of YS1646 were observed after 16 hours of incubation at 37°C in 5% CO<sub>2</sub> (Figure 1). However, by plating more cells, the presence of a few resistant colonies could be detected, as we obtained  $3.3 \times 10^8$  CFU/ml on plates incubated in air and  $1.7 \times 10^5$  CFU/ml on plates incubated in the presence of 5% CO<sub>2</sub>, a greater than 3 log reduction. This CO<sub>2</sub> sensitivity, first observed in YS1646, is also observed in a simple *msbB* mutant (see below). In contrast, wild-type *Salmonella* Typhimurium (ATCC 14028 and LT2), *Salmonella* Typhi (CS029, ATCC 33458), and *Escherichia coli* (MG1655, near-wild type K-12) are resistant to 5% CO<sub>2</sub> (ATCC 14028: Figure 1; other strains: data not shown). Interestingly, *msbB* *E. coli* (KL423) was not sensitive to CO<sub>2</sub> (not shown), consistent with there being physiologically relevant differences between the *E. coli* and *Salmonella* in regard to the loss of MsbB function, as has been previously observed [4]. These differences obscure or compensate for obvious growth defects in *msbB* *E. coli*.

CO<sub>2</sub> sensitivity was found in all *msbB* *Salmonella* strains tested so far, indicating that CO<sub>2</sub> sensitivity is a direct result of the lack of lipid A myristoylation (data not shown, see list of strains in Table 1). Consistent with these results, normal growth in CO<sub>2</sub> was completely restored when *msbB* was expressed from a plasmid (pSM21(*msbB*<sup>+</sup>)) (see Table 1).

### The *somA* (for EGTA and salt resistance) and Suwwan deletion (for EGTA, salt, and galactose-MacConkey resistance) *msbB* suppressors do NOT suppress sensitivity to 5% CO<sub>2</sub>

Two *msbB* *Salmonella* strains with secondary mutations that allow faster growth are YS873 and YS1646. YS873 has a loss-of-function mutation in *somA* [4] and YS1646 has a large deletion, referred to as the Suwwan deletion [9], that includes *somA* plus ~100 other genes. The *somA* mutation in YS873 suppresses growth defects on EGTA and salt-containing media [4] and the Suwwan deletion in YS1646



**Figure 1**  
**Sensitivity and resistance to CO<sub>2</sub> shown by comparing colony forming units (CFUs).** Each strain was grown overnight in LB broth and diluted 10<sup>6</sup> fold, and then 100 μl was spread on each plate and incubated. Upper panel: wild type *Salmonella* (14028) on LB media in air (left) or 5% CO<sub>2</sub> (right). Lower panel; YS1646 on LB media in air (left) or 5% CO<sub>2</sub> (right).

suppresses sensitivity to EGTA, salt, and galactose MacConkey media [9]. However, neither the *somA* mutation nor the Suwwan deletion suppresses MsbB-mediated sensitivity to 5% CO<sub>2</sub> (Suwwan deletion in YS1646, Figure 1; *somA* in YS873, see below). As shown in Figure 1, when plating identical dilutions containing greater than 100 CFU onto LB agar from an MSB broth culture of YS1646 and wild type *Salmonella*, no YS1646 colonies are detected after 24 hours of incubation in 5% CO<sub>2</sub> at 37°C. Since we have not yet identified all of the genes within the Suwwan deletion that are responsible for the suppressor phenotype, we focused our study on YS873, which has clearly defined mutations in *msbB* and *somA*.

#### **CO<sub>2</sub> resistant mutations are detected at high frequency in *msbB somA Salmonella***

Subsequent experiments revealed that spontaneous CO<sub>2</sub> resistant mutants are detected when higher numbers of YS873 bacteria are plated and incubated under 5% CO<sub>2</sub> conditions. The mutation frequency of spontaneous CO<sub>2</sub> mutants from an MSB broth culture was determined to be ~3 out of 10<sup>4</sup> (not shown), which is similar to the frequency that EGTA and galactose MacConkey suppressor mutations arise in *msbB Salmonella* [4].

#### **A loss-of-function mutation in *zwf* suppresses CO<sub>2</sub> sensitivity**

In our preliminary studies, several spontaneous CO<sub>2</sub> resistant mutants were isolated that showed a high degree

of instability. Therefore, we subsequently focused on the use of Tn5 mutagenesis, which is known to generate stable insertions primarily associated with null mutations. To screen for a mutation that would compensate for CO<sub>2</sub> sensitivity, a random Tn5 insertion library of YS1646 was created and selected on LB agar in 5% CO<sub>2</sub>. 9 clones were isolated, of which we determined the insertion sites in three of the clones using a genome-walking method. All of the Tn5 insertions identified were located in the monocistronic *zwf* gene. Two of the insertions (clones 14.2 and 32.2) were identical (possible siblings), located after open reading frame nucleotide 1019, and the third (clone 37.2) was located at after base pair 1349. Because we focused our screening on Tn5 insertions, we do not know if other mutagenesis methods would have isolated clones with mutations in other genes. *zwf* encodes glucose-6-phosphate-dehydrogenase, an enzyme of the pentose-phosphate-pathway (PPP). In this pathway, Zwf converts glucose-6-phosphate, from glycolysis, to 6-phosphogluconate, generating NADPH + H. The subsequent reaction, catalyzed by Gnd, converts 6-phosphogluconate to ribulose-5-phosphate, generating NADPH + H and CO<sub>2</sub> (Figure 2). A non-polar deletion (see materials and methods) was created in *zwf* ( $\Delta zwf82$ ) using the pCVD442 vector [10] to test if the phenotypes arise from loss of the *zwf* gene or a polarity effect. The *zwf* non-polar deletion was found to exhibit the same CO<sub>2</sub> growth phenotypes as the *zwf* Tn5 insertions. Subsequent experiments use the non-polar deletion in *zwf* in 14028 and YS873. A loss-of-function mutation in *zwf* results in smaller colony size than *zwf*<sup>+</sup> strains on agar media in both wild type and *msbB* genetic backgrounds.

#### **Gluconate prevents suppression of CO<sub>2</sub> sensitivity by *zwf***

Zwf catalyzes the first step of the pentose phosphate pathway (PPP). PPP produces NADPH for anabolic pathways and the molecules generated by this pathway serve as building blocks for nucleotides, sugars, amino acids, and vitamins [11]. As shown in Figure 2, Zwf catalyzes the conversion of glucose-6-phosphate to 6-phosphogluconate. 6-phosphogluconate can also be formed from gluconate by gluconate kinase [12], which bypasses the PPP's requirement for Zwf (Figure 2). The addition of gluconate to media thereby allows for the production of 6-phosphogluconate in the absence of Zwf. The enzyme gluconate-6-phosphate dehydrogenase (Gnd) then decarboxylates 6-phosphogluconate, converting it from a 6-carbon to a 5-carbon (ribulose-5-phosphate) sugar and releasing CO<sub>2</sub> gas. Perhaps a threshold of CO<sub>2</sub> must be passed to inhibit the growth of *msbB Salmonella* and a loss-of-function mutation in *zwf* allows for the CO<sub>2</sub> level to remain below this threshold. Previous reports of *zwf E. coli* show reduced CO<sub>2</sub> production when grown in minimal media with acetate or pyruvate as a carbon source. However, *zwf E. coli* produced more CO<sub>2</sub> than wild type when grown in minimal media with glucose [13,14]. Further

**Table 1: Bacterial strains and plasmids**

Strain or plasmid	Parental strain	Genotype	Derivation or source
<i>S. enterica</i> serovar Typhimurium			
I4028	I4028	Wild type	ATCC I4028
I4028 $\Delta$ zwf	I4028	$\Delta$ zwf82	Replacement of zwf gene with $\Delta$ zwf82 by homologous recombination
I4028 gnd	I4028	<i>gnd-189::MudJ</i> (Kan <sup>R</sup> )	P22-DM4483 $\times$ I4028 $\rightarrow$ Kan <sub>40</sub> <sup>R</sup>
YS1646(=VNP20009)	I4028	$\Delta$ msbB2 $\Delta$ purl $\Delta$ Suwwan	[9,31]
VNP20057	YS1646	$\Delta$ msbB2 $\Delta$ purl <i>zwf81::Tn5</i> (Kan <sup>R</sup> ) $\Delta$ Suwwan	YS1646 $\times$ P22 Tn5 pool (on I4028) $\rightarrow$ selection on LB plates in 5% CO <sub>2</sub>
YS1	I4028	<i>msbB1::<math>\Omega</math>tet</i>	[4]
YS1 <i>msbB</i> *	YS1	<i>msbB1::<math>\Omega</math>tet/pSM21<i>msbB</i>*</i> (Amp <sup>R</sup> )	Plasmid pSM21 [4] into YS1
YS1 <i>zwf</i> (=YS11)	YS1	<i>msbB1::<math>\Omega</math>tet zwf:Tn5</i> (Kan <sup>R</sup> )	P22-VNP20057 $\times$ YS1 $\rightarrow$ Kan <sub>20</sub> <sup>R</sup>
YS873	I4028	<i>msbB1::<math>\Omega</math>tet somA1 zbj10:Tn10</i>	[4]
YS873 <i>msbB</i> * (=YS8731)	YS873	<i>msbB1::<math>\Omega</math>tet somA1 zbj10:Tn10/pSM21<i>msbB</i>*</i> (Amp <sup>R</sup> )	Plasmid pSM21 [4] into YS873
YS873 <i>zwf</i> (=YS8732)	YS873	<i>msbB1::<math>\Omega</math>tet somA1 zbj10:Tn10 zwf81:Tn5</i> (Kan <sup>R</sup> )	P22-VNP20057 $\times$ YS873 $\rightarrow$ Kan <sub>20</sub> <sup>R</sup>
YS873 $\Delta$ zwf (=YS8733)	YS873	<i>msbB1::<math>\Omega</math>tet somA1 zbj10:Tn10 <math>\Delta</math>zwf82</i>	Replacement of <i>zwf81::Tn5</i> gene in YS873 <i>zwf</i> with $\Delta$ zwf82 by homologous recombination
YS873 <i>gnd</i> (=YS8734)	YS873	<i>msbB1::<math>\Omega</math>tet somA1 zbj10:Tn10 gnd-189::MudJ</i> (Kan <sup>R</sup> )	P22-DM4483 $\times$ YS873 $\rightarrow$ Kan <sub>10</sub> <sup>R</sup>
LT2	LT2	Wild type	ATCC 15277
DM4483	LT2	<i>gnd-189::MudJ</i> (Kan <sup>R</sup> )	Gift of Diana Downs and Eugene I. Vivas, U. of Wisconsin
YS501	LT2	<i>recD541::Tn10dCm hsdSA29 hsdSB121 hsdL6 metA22 metE551 trpC2 ilv-452 H1-b H2-e,n,x fla-66 nml(-) rpsL120 xyl-404 galE719</i>	[5]
<i>Salmonella enterica</i> serovar Typhi CS029			
<i>Salmonella enterica</i> serovar Typhi ATCC 33458			
<i>E. coli</i> K-12 MG1655	MG1655	F-1- <i>rph-1</i>	[32]
KL423	MG1655	F-1- <i>rph-1 msbB1::<math>\Omega</math>Cm</i>	[4]
pCVD442		Amp <sup>R</sup>	[10]
pCVD442 $\Delta$ zwf82		Amp <sup>R</sup>	This study
pSP72		Amp <sup>R</sup>	Promega Corporation
pSP72 <i>lacZ</i>		<i>lacZ</i> , Amp <sup>R</sup>	This study
pSM21		<i>msbB</i> , Amp <sup>R</sup>	[4]

studies will be required to clarify the production of CO<sub>2</sub> by *Salmonella* grown in Luria-Bertani-based media and its contribution to CO<sub>2</sub> sensitivity.

To test whether *zwf*'s suppressive effects result from its role in PPP pathway products and not from some unknown function, we observed the effect of gluconate on CO<sub>2</sub> sensitivity in our mutants. Growth of YS873 *zwf* was tested on LB-0 plates containing 0.33% gluconate in ambient air and 5% CO<sub>2</sub> (Figures 3I and 3J). As we hypothesized, YS873 *zwf* was not able to grow on LB-0 gluconate in 5% CO<sub>2</sub>. Thus, we confirmed that the *zwf*'s suppression of CO<sub>2</sub> sensitivity results from its known enzymatic step in the PPP pathway. We also found a new phenotype for unsuppressed *msbB* *Salmonella*: YS1 does not grow on LB-0 agar in the presence of 0.33% gluconate (Figure 3I). To test if the production of 6-phosphogluconate or a downstream PPP metabolite is responsible for mediating CO<sub>2</sub> resistance, we tested for CO<sub>2</sub> resistance in a YS873 *gnd-189::MudJ* mutant (*Gnd* catalyzes the second step of the PPP pathway, Figure 2) and found that the strain remained CO<sub>2</sub> sensitive (data not shown). Therefore, we conclude that the production of 6-phosphogluconate, by either *Zwf* or gluconate kinase, contributes to CO<sub>2</sub> sensitivity in an *msbB* genetic background.

#### ***zwf* mutation suppresses both *msbB*-induced CO<sub>2</sub> sensitivity and osmotic defects**

For further analysis of the *msbB zwf* phenotype, the *zwf* (*zwf81::Tn5*) mutation was transduced into *msbB* (YS1) and *msbB somA* (YS873) genetic backgrounds to generate strains YS1 *zwf* and YS873 *zwf* respectively. As shown in the replica plate series of Figure 3, growth of unsuppressed YS1 is inhibited on LB (Figure 3A) and LB-0 gluconate (Figure 3I) but it grew well on MSB and LB-0 agar (Figures 3C and 3E), confirming the results of Murray et al. [4]. In contrast, growth of YS1 on MSB and LB-0 agar is completely inhibited when the plates are incubated in the presence of 5% CO<sub>2</sub>. The introduction of the *zwf* mutation completely compensates for the phenotype and allows the

bacteria to grow under 5% CO<sub>2</sub> on all three media (Figures 3B, 3D and 3F). However, it does not rescue YS1 from gluconate sensitivity (Figure 3I).

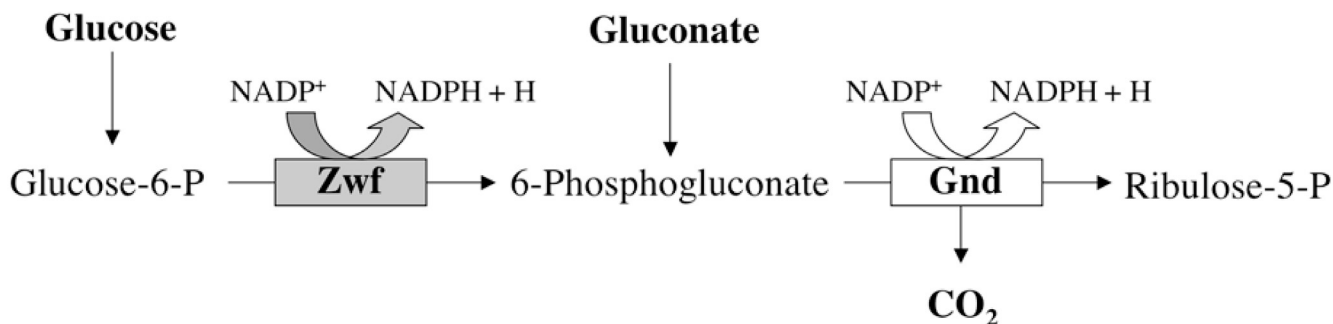
When NaCl in LB plates is substituted with sucrose at iso-osmotic concentrations (Figures 3G), growth of YS1 is also inhibited, indicating osmosensitivity of YS1. Interestingly, introduction of the *zwf* mutation improves growth of YS1 on LB and on LB-0 5% sucrose agar, indicating that the *zwf* mutation can partially compensate for the *msbB*-induced osmotic growth defect.

MSB media contains high levels of divalent cations, which have been proposed to increase lateral interactions between the phosphate groups of neighboring lipid A molecules [15]. Based on Murray et al.'s finding [16] that a decrease in electrostatic repulsion between the phosphates of lipid A can help to compensate for the lack of the myristic acid residue, we investigated whether Mg<sup>2+</sup> and Ca<sup>2+</sup> would protect against the detrimental effects of 5% CO<sub>2</sub>. On agar plates, Mg<sup>2+</sup> and Ca<sup>2+</sup> showed partial protection in YS873 (Figure 3D).

YS873, which contains the EGTA and salt resistance suppressor mutation *somA* [4], grows well on LB (Figure 3A), MSB (Figure 3C), LB-0 (Figure 3E) and LB-0 sucrose (Figure 3G) agar plates in air, but not when the plates are incubated in 5% CO<sub>2</sub> (Figures 3B, 3D, 3F, and 3H). In contrast, the strain YS873 *zwf* is able to grow on all of these media in CO<sub>2</sub>, indicating that the *zwf* mutation can compensate for the growth defect of *msbB* strains in CO<sub>2</sub> (Figure 3). Subsequent experiments were performed using the YS873 (*msbB somA*) genetic background because unsuppressed *msbB* *Salmonella* can not grow under mammalian physiological salt conditions [4].

#### ***msbB somA* *Salmonella* are sensitive to CO<sub>2</sub> in LB and LB-0 broth**

Figure 4 shows the growth of wild type ATCC 14028, 14028 *zwf*, YS873, and YS873 *zwf* in LB and LB-0 broth,



**Figure 2**  
Steps of the Pentose Phosphate Pathway (PPP) highlighting the relationship of the *Zwf* enzyme, gluconate, and *Gnd*-based production of CO<sub>2</sub>.

incubated in the presence or absence of 5% CO<sub>2</sub>. As shown in Figure 4, the growth of YS873 (Figure 4A), but not ATCC 14028 (Figure 4C) is greatly impaired in LB broth in the presence of 5% CO<sub>2</sub>. A significant decrease in CFU is observed (Figure 4A), indicating that YS873 cells lose viability in the presence of 5% CO<sub>2</sub> in LB broth. When a loss-of-function mutation in *zwf* is incorporated into YS873, no loss in viability is observed under identical conditions, although there is a longer lag phase of growth (Figure 4A). In LB-0 broth, there are no growth defects in 14028 or 14028 *zwf* (Figure 4D). For YS873 and YS873 *zwf*, the growth defects in LB-0 in the presence of 5% CO<sub>2</sub> are attenuated in comparison to those observed in LB broth. There is no decrease in viability in YS873 in LB-0 in 5% CO<sub>2</sub>, although there is impaired growth in both YS873 and YS873 *zwf* in LB-0 in the presence of CO<sub>2</sub> compared to growth in the absence of CO<sub>2</sub> (Figure 4B).

**YS873 has severe morphological defects in LB broth under 5% CO<sub>2</sub> conditions that are suppressed by a loss-of-function mutation in *zwf***

Since our results show that *msbB* *Salmonella* lose viability in the presence of 5% CO<sub>2</sub> (Figure 4), we examined *msbB* mutants grown in the presence of 5% CO<sub>2</sub> to determine if there are any defects in cell morphology or chromosome segregation. Differential interference contrast (DIC) microscopy shows striking morphological defects under CO<sub>2</sub> conditions (Figure 5K), with long, bulging filamentous YS873 cells. DAPI staining shows no apparent chromosomal segregation defects, as no cells lacking DNA were observed (Figure 5L). However, the cell directly under the "K" and "L" labels appears to be lysing (see thick arrow).

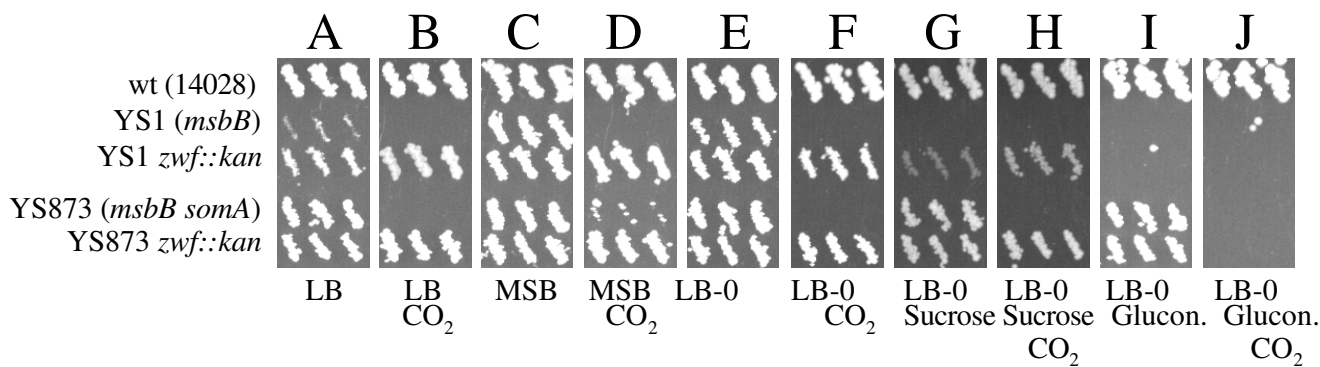
As shown in Figures 5O and 5P, *zwf* suppresses the severe morphological defects in YS873 grown in LB in the presence of 5% CO<sub>2</sub>. Many cells are elongated but lack gross morphological defects. Growth in LB in a 5% CO<sub>2</sub> environment caused wild type ATCC 14028 *Salmonella* to form minicells, with minicells (see thin arrows) accounting for ~15% of the cells (21/144) (Figure 5C and 5D as compared to Figures 5A and 5B). As seen in Figure 5E and 5F, 14028 *zwf* exhibits ~21% minicell formation in LB broth, even without CO<sub>2</sub> (20/95 cells). Thus, we conclude that both CO<sub>2</sub> and *Zwf* can, either directly or indirectly, affect cell division.

***β*-galactosidase assays confirm cell lysis in LB in the presence of 5% CO<sub>2</sub>**

Microscopy (Figure 5K and 5L) suggested that some YS873 cells were lysing in LB in the presence of 5% CO<sub>2</sub>. To test if the decrease in CFU observed in YS873 in LB in the presence of 5% CO<sub>2</sub> resulted from cell lysis, a plasmid expressing *β*-galactosidase was electroporated into YS873 and YS873 *zwf* and the cells were grown in LB in the presence or absence of CO<sub>2</sub>. As shown in Figure 6, after 6 hours of growth, significant cell lysis is observed in YS873 grown in the presence of 5% CO<sub>2</sub> as measured by the release of the cytoplasmic enzyme *β*-galactosidase. Furthermore, a loss-of-function mutation in *zwf* significantly reduces cell lysis in YS873. No significant cell lysis is observed in the absence of CO<sub>2</sub>.

**CO<sub>2</sub> sensitivity does not result from increased acidification of LB media and *zwf* suppresses sensitivity to acidic pH in LB broth**

During this study, we observed that the pH of LB broth dropped from pH 7.0 to pH 6.6 after equilibration in 5%



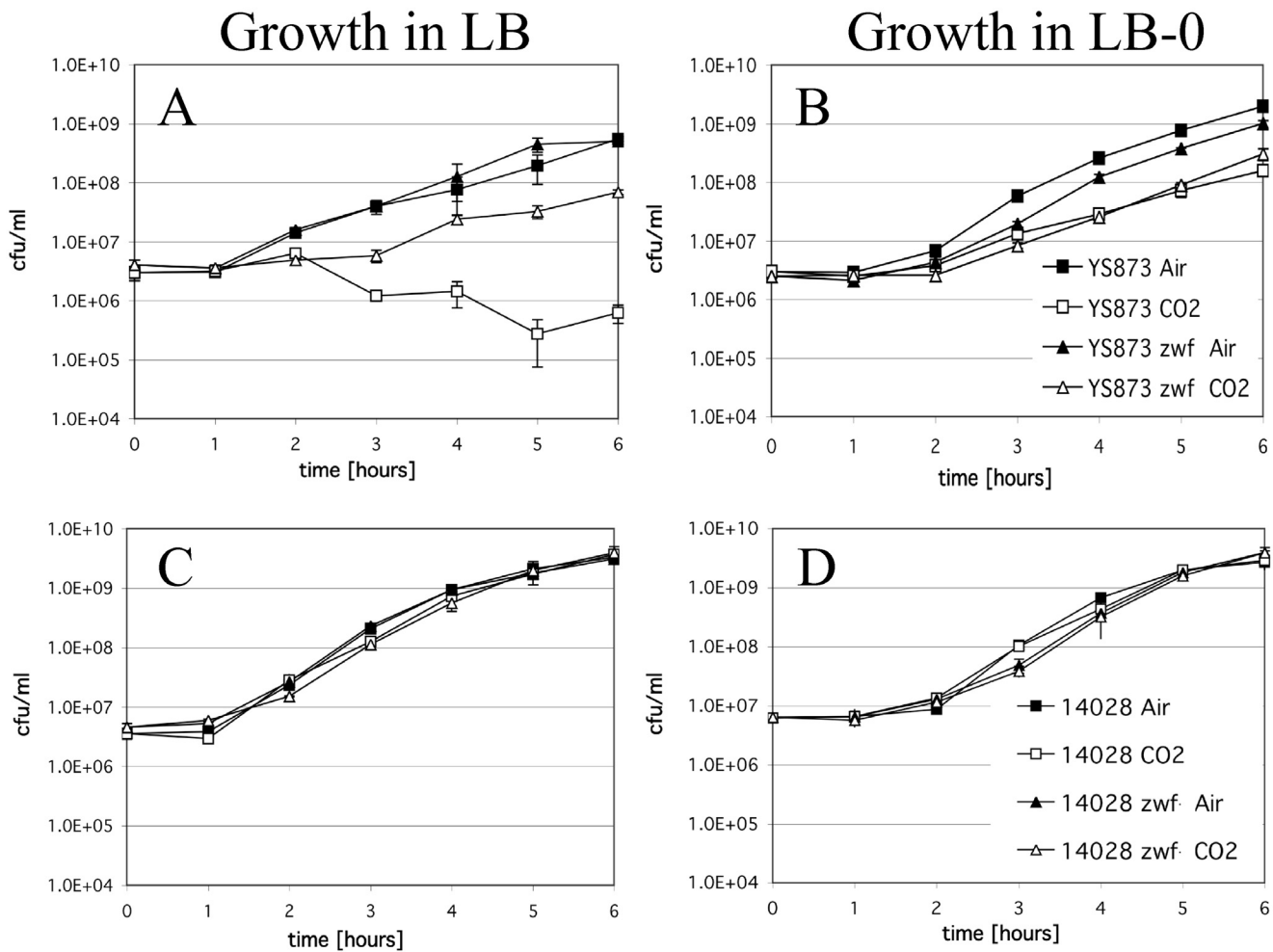
**Figure 3**  
***zwf* mutation suppresses both *msbB*-induced CO<sub>2</sub> sensitivity and osmotic defects.** Double velvet replica plates with different media were used to indicate the ability of small patches of bacteria (3 each) to grow. The strains used are listed on the left. Growth conditions (all at 37°C) included: A, LB media in air; B, LB media in 5% CO<sub>2</sub>; C, MSB media in air; D, MSB media in 5% CO<sub>2</sub>; E, LB-0 media in air; F, LB-0 media in 5% CO<sub>2</sub>; G, LB-0 media containing sucrose (total 455 miliosmoles) in air; H, LB-0 media containing sucrose in 5% CO<sub>2</sub>; I, LB-0 + gluconate (glucon.) in air; J, LB-0 + gluconate in 5% CO<sub>2</sub>.

CO<sub>2</sub>. Since CO<sub>2</sub> can acidify bicarbonate buffered media, we tested whether part of the CO<sub>2</sub> sensitivity was due to acidification of the media. Thus, to test if increased or decreased pH would alter sensitivity to CO<sub>2</sub> in LB broth, we buffered LB broth to pH 7.6, or 6.6, and cultures were grown in the presence or absence of 5% CO<sub>2</sub>. As shown in Figure 7, wild type ATCC 14028 and ATCC 14028 *zwf* grow normally under all conditions in LB broth in the absence (Figure 7C) or presence (Figure 7D) of 5% CO<sub>2</sub>. In contrast, the growth of YS873 is significantly impaired when the pH of LB is 6.6, with no significant increase in CFU after 6 hours (Figure 7A), whereas when the pH of LB is 7.6, YS873 grows well (Figure 7A). A loss-of-function mutation in *zwf* allows for YS873 to grow well in LB broth at a pH of 6.6 (Figure 7A). 5% CO<sub>2</sub> inhibited the growth of YS873 and YS873 *zwf* in LB pH 6.6 and 7.6 (Figure 7B). Although *zwf* protects against 5% CO<sub>2</sub> in LB broth pH 6.6

(Fig 7B), it does not significantly improve survival in the presence of 5% CO<sub>2</sub> in LB broth pH 7.6 (Figure 7B), suggesting that an acidic pH is a component for *zwf* to suppress *msbB*-mediated sensitivity to 5% CO<sub>2</sub>.

***β*-galactosidase assays confirm cell lysis in LB broth, pH 6.6, in air**

To test if the loss of growth of YS873 in LB broth pH 6.6 was the result of cell death or simply the result of inhibition or delay of cell division, *β*-galactosidase release was measured. As shown in Figure 8A, significant cell lysis occurs after growth of YS873 for 8 hours in LB broth, pH 6.5 but not pH 7.5 (pH shifted slightly [±0.1 units] during autoclaving). Furthermore, a loss-of-function mutation in *zwf* significantly reduces cell lysis of YS873 grown in LB broth pH 6.5. This reduction in cell lysis, as measured by release of the cytoplasmic enzyme *β*-galactosi-



**Figure 4**  
***msbB* confers growth sensitivity in liquid media under CO<sub>2</sub> conditions containing physiological amounts of salt and this is suppressed by *zwf*.** Two sets of *Salmonella* strains (YS873 and YS873 *zwf*; 14028 and 14028 *zwf*) were grown on either LB (A and C) or LB-0 (B and D) in either air or 5% CO<sub>2</sub>.

dase, correlates with increased CFU/ml numbers observed in YS873 *zwf* (as compared to YS873) grown in LB broth, pH 6.6 (Figure 7A).

#### ***zwf* reduces YS873 cell lysis in the presence of 5% CO<sub>2</sub> in LB broth pH 6.6, but not pH 7.6**

Since we observed that YS873 lysed when there was no net growth in LB broth pH 6.5 while maintaining a relatively constant CFU/ml, we investigated if cell lysis occurs in YS873 *zwf*, which also exhibits little net growth with a relatively constant CFU/ml in the presence of 5% CO<sub>2</sub> in LB broth pH 6.6 or 7.5 (Figure 7B). Growth curves for these strains indicated that there was a decrease in CFU/ml when YS873 was grown in LB broth pH 6.6 in the presence of 5% CO<sub>2</sub>, but that CFU/ml remained relatively constant if a loss-of-function mutation in *zwf* was present or if the pH of LB broth was 7.5 (Figure 7B). Figure 8 (8 hours) shows that significant cell lysis, as indicated by release of the cytoplasmic enzyme β-galactosidase, occurs when YS873 is grown in the presence of 5% CO<sub>2</sub> at pH 6.6 or 7.6, and in YS873 *zwf* grown in the presence of 5% CO<sub>2</sub> in LB pH 7.5. YS873 *zwf* exhibited significantly less lysis in the presence of 5% CO<sub>2</sub> in LB broth pH 6.6, showing that a loss-of-function mutation in *zwf* significantly suppresses sensitivity to CO<sub>2</sub> at neutral (as shown in Figure 6) or slightly acidic pH (Figure 8B). Again, we found that significant cell lysis can occur with a relatively constant CFU/ml (Figure 8B: YS873 *zwf* in LB pH 7.6).

## **Discussion**

### ***msbB* Salmonella pleiotropy**

The *msbB* gene was mutated to reduce the toxicity of *Salmonella* in mice and humans [5,6]. In order for these strains to function within mammalian systems they must be able to persist under normal mammalian physiological conditions. In contrast to other reports [17-20], we found *msbB* *Salmonella* to have striking growth defects, demonstrating sensitivity to salt, EGTA, MacConkey media, and polymyxin B sulfate [4,9,16]. Here we report additional sensitivity to osmolarity, gluconate, acidic pH and 5% CO<sub>2</sub> growth conditions. Significantly, *msbB* *Salmonella* are sensitive to the conditions found within mammals, where blood has significant levels of salt and CO<sub>2</sub>; we therefore we screened for a suppressor of *msbB*-associated CO<sub>2</sub> sensitivity.

### ***zwf* suppresses CO<sub>2</sub> sensitivity in *msbB* Salmonella**

Glucose-6-phosphate-dehydrogenase (encoded by *zwf*) catalyzes the first enzymatic step in the pentose phosphate pathway (PPP), which converts glucose-6-phosphate to 6-phosphogluconate and NADPH + H. In *E. coli*, *zwf* is regulated by several mechanisms including anaerobic growth [21], growth rate [22], weak acids as well as superoxide [23]. Weak acids appear to regulate *zwf* through the multiple antibiotic resistance (*mar*) regulon, whereas superoxide exposure induces *zwf* through the Sox R/S regulon

and contributes to DNA repair [24]. *zwf* mutants of *Pseudomonas* are hypersensitive to superoxide generating agents such as methyl viologen [25].

*Salmonella* Typhimurium *zwf* might be regulated by a different set of environmental signals than *E. coli*. Superoxide, while clearly activating other SoxR/S regulated genes like *sodA* and *fumC*, does not induce *zwf* transcription [26]. *S. Typhimurium* *zwf* mutants have been shown to be less virulent in mice and more sensitive to reactive oxygen and nitrogen intermediates [27]. In general, it is thought that the expression of *zwf* and subsequent generation of NADPH helps cells to combat oxidative stress. Interestingly, SoxS mutants of *Salmonella* are not attenuated in mice [28], suggesting that even though *zwf* expression is important for survival, superoxide generated responses might not be required. In the case of *msbB* mutants, the *zwf* mutation restores wild type growth under 5% CO<sub>2</sub> and pH 6.5 conditions, suggesting that the expression of *zwf* is detrimental for growth of *msbB* mutants in an acidic or increased CO<sub>2</sub> atmosphere. Furthermore, our data showing that a loss-of-function mutation in *gnd* (which produces the second enzyme of the PPP pathway, Figure 2) does not suppress sensitivity to CO<sub>2</sub> suggests that the production of 6-phosphogluconate, by either Zwf or gluconate kinase, contributes to CO<sub>2</sub> sensitivity in *msbB* *Salmonella*.

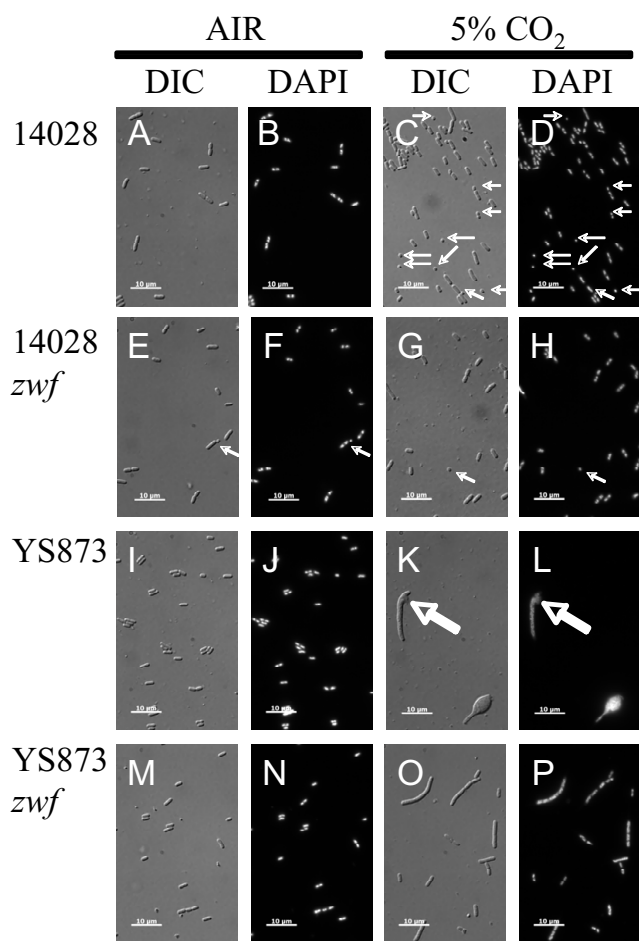
### ***MsbB* as a virulence factor?**

Several publications cite *MsbB* as a virulence factor that is necessary for both septic shock and the ability to invade and persist in mammalian cells [5,17,29]. However, owing to the fact that *msbB* *Salmonella* were tested under 5% CO<sub>2</sub> conditions, the lack of virulence may be partially or fully due to the inability of *msbB* *Salmonella* to grow in the presence of the 5% CO<sub>2</sub>. Further experimentation with *msbB zwf* *Salmonella* will be necessary to determine which virulence defects are attributable to *msbB* lipid A and those that arise from sensitivity to 5% CO<sub>2</sub>. Based upon this study and earlier studies on the sensitivity of *zwf* mutant to superoxides, *zwf* may both reduce virulence on one hand, yet potentiate growth under CO<sub>2</sub> conditions on the other, further complicating virulence analyses.

## **Conclusion**

Here, we report new growth defects in *msbB* *Salmonella*: sensitivity to gluconate and growth in hypertonic, acidic or 5% CO<sub>2</sub> conditions. These characteristics are in addition to the previously reported growth defects in the presence of salt, EGTA, polymyxin, or MacConkey media. Previous studies showing that *MsbB* is a virulence factor require further evaluation of the role that CO<sub>2</sub> sensitivity plays. The potential for cryptic, spontaneous mutations remains a possibility that should be addressed by re-transduction under non-selective conditions followed by plating independently under CO<sub>2</sub> and ambient air. We have





**Figure 5**  
**YS873 has severe morphological defects in LB broth under 5% CO<sub>2</sub> conditions that are suppressed by a loss-of-function mutation in *zwf*.** DIC, Differential Interference Contrast; DAPI, 4',6'-diamidino-2-phenylindole (DNA stain); Thick arrows point to lysis; Thin arrows point to minicells.

created an *msbB somA zwf* *Salmonella* strain that is resistant to growth under acidic or 5% CO<sub>2</sub> conditions. This strain contains a loss-of-function mutation in *zwf*, an enzyme in the pentose phosphate pathway that produces CO<sub>2</sub> as it converts a 6 carbon sugar to a 5 carbon sugar. The study of the virulence of *msbB zwf* *Salmonella* will allow the determination of what types of virulence are attributable to cells having an MsbB lipid A independent of sensitivity to 5% CO<sub>2</sub>, which is required for *in vitro* and *in vivo* virulence assays.

## Methods

### Bacterial strains, plasmids, phage and media

The bacterial strains and plasmids used in this study are listed in Table 1. The *Salmonella msbB* insertion/deletion

for tetracycline resistance was described by Low et al. [5]. P22 mutant HT105/1int201 (obtained from the *Salmonella* Genetic Stock Center, Calgary, Canada) was used for *Salmonella* transductions. *Salmonella enterica* serovar Typhimurium strains were grown on LB-0 or MSB agar or in LB, LB-0, buffered LB or MSB broth. MSB media consists of LB (Luria-Bertani media, [30]) with no NaCl and supplemented with 2 mM MgSO<sub>4</sub> and 2 mM CaCl<sub>2</sub>. LB-0 is LB media with no NaCl. Buffered LB pH 7.5 and pH 6.5 consisted of LB-0 with 100 mM NaPO<sub>4</sub> adjusted to 455 mOsmol by adding NaCl. MSB broth and agar were used for the growth of strains under non-selective conditions. LB-0 agar was used when using selective antibiotics in transductions and transformations. Plates were solidified with 1.5% agar. LB-0 agar or MSB broth were supplemented as needed with ampicillin (100 µg/ml) or kanamycin (20 µg/ml). Antibiotics were added to LB-0 agar after cooling to 45 degrees Celsius.

### Restoring *msbB*<sup>+</sup> genotype

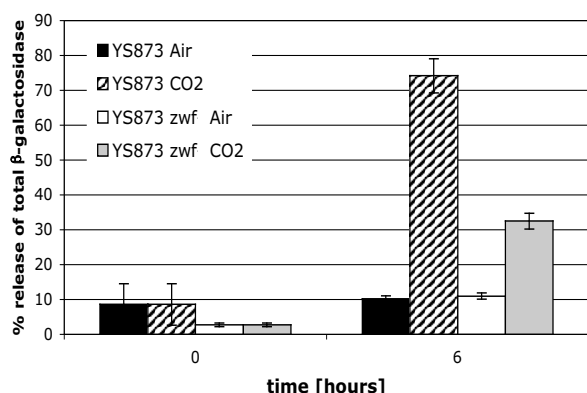
In order to confirm that the observed CO<sub>2</sub> sensitivity results simply from knocking out MsbB function, wild type *msbB* was expressed from the *msbB* promoter using plasmid pSM21 [4]. Purified plasmids were transformed into electroporation-competent cells of strains YS1 and YS873.

### Growth Analysis

Phenotypes of strains were determined by replica plating. Master plates were made on either MSB or LB-0 agar. Replica plating was performed using a double velvet technique [4]. Replica plates were incubated for 16 hours at 37°C. To generate growth curves, 3 ml broth tubes were inoculated with single colonies and grown on a shaker overnight at 37°C in air. Cells were diluted 1:1000 or 1:500 (β-gal strains) in LB broth. Cells were held on ice until all inoculations were completed. Triplicate cultures were then placed in a 37°C shaker with 250 rpm in air or 5% CO<sub>2</sub>. O.D.<sub>600</sub> was measured every 60 minutes and dilutions of bacteria were plated onto MSB or LB agar plates to calculate the number of colony forming units (CFU) per ml.

### Microscopic Observation

Strains 14028, 14028 *zwf*, YS873 and YS873 *zwf* were grown for 6 hours, as described above for growth curves, at 200 RPM. The cells were then fixed for microscopy using a solution of 30 mM sodium phosphate buffer (pH 7.5) and 2.5% formaldehyde. Cell morphology was observed with a Zeiss Axiovision microscope using differential interference contrast settings and DNA was detected via DAPI fluorescence. Fixed cells were incubated with 2 µg/ml DAPI for 10 minutes in the dark and aliquoted onto a 1% agarose pad.



**Figure 6**  
 $\beta$ -galactosidase release assays confirm cell lysis in LB in the presence of 5% CO<sub>2</sub> and that *zwf* confers resistance. Release of  $\beta$ -galactosidase from the cytosol of the bacteria was used to test if the decrease in CFU observed in YS873, in LB in the presence of 5% CO<sub>2</sub>, resulted from cell lysis. The strains were grown under either ambient air or 5% CO<sub>2</sub> conditions.

#### Mutation Frequency Determination

A frozen stock of YS873 was streaked on MSB media and incubated overnight at 37°C to isolate individual clones. Triplicate 3 ml of LB broth were inoculated with independent YS873 colonies. They were grown at 37°C in a shaker over night. The tubes were then placed on ice and diluted in 0.9% saline. 10<sup>-6</sup> and 10<sup>-4</sup> dilutions were plated in duplicates onto LB agar and incubated in air and CO<sub>2</sub> incubators respectively overnight at 37°C to calculate the number of CFU per ml.

#### Transduction and Transformation

*Salmonella* P22 transductions were performed by the method of Davis et al. [30], except that LB-0 plates supplemented with the appropriate antibiotic were used. EGTA was not added to the antibiotic plates for transductions. A BioRad Gene Pulser was used for electroporation with the following settings: 2.5 kV, 1000 ohms and 25  $\mu$ FD for transformation of YS1 and 1.7 kV, 186 ohms and 25  $\mu$ FD were used for YS873, YS1646, and ATCC 14028 [4].

#### Tn5 mutagenesis and mapping

A library of transposons in YS1646 was made using the EZ::TN <Kan-2> insertion kit from Epicentre (Madison, WI). Over 56,000 kanamycin resistant (Kan<sup>R</sup>) clones of YS1646 were pooled. The pool was screened for mutation rate and auxotrophy for different biosynthetic pathways by replica plating onto minimal media and media containing various pools of amino acids and bases [30]. Following selection for CO<sub>2</sub> resistance by plating dilutions to

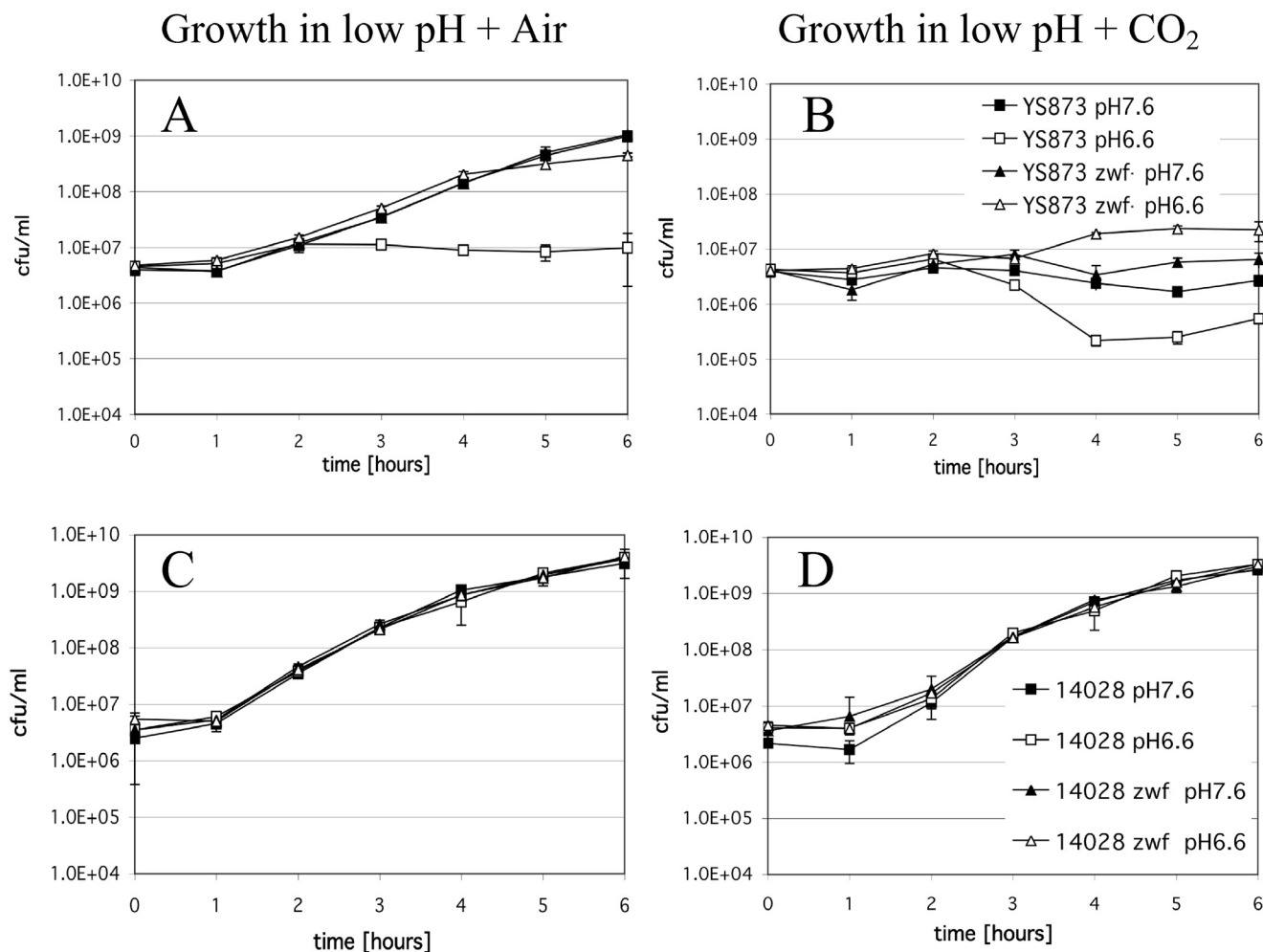
LB-Kan and incubating in 5% CO<sub>2</sub>, the colonies were again pooled and a P22 lysate was generated and transduced to a non-suppressed strain and purified for kanamycin resistance under non-CO<sub>2</sub> conditions in order to separate spontaneous mutants from Tn5-based suppressors. Transposon-associated Tn5 insertions were identified by replica plating in air and CO<sub>2</sub>. Mapping of the insertion sites was performed by using the Genome-Walker™ kit (Clontech, Mountain View, CA) according to the manufacture's instructions.

#### Construction of non-polar deletion in *zwf*

A non-polar deletion in *zwf* was generated by constructing a pCVD442 vector capable of deleting the entire *zwf* coding region by homologous recombination with the *Salmonella* chromosome [10]. Primers for PCR were designed that would generate one product immediately upstream of the 5' ATG start codon and a separate product immediately downstream of the 3' stop codon of the *zwf* coding region. The two separate products could then be ligated sequentially into the pCVD442 vector. The primers were: *zwf*-5'-reverse: 5'-GTGTGAGCTCGTGGCTTCGCGCGC CAGCGG CGTCCAGC-3' (with added *Sac*I), *zwf*-5'-forward: 5'-GTGTGCATGCGGGGGG CCATATA GG CCGGGGATTTAAATGTCATTCTCCTTAGTTAATCTCCTG G-3' (with added *Sph*I), *zwf*-3' reverse: 5'-GTGTGCATGCGGGGTTAATTAA GGGGGCGGCCGATTTGCCACTCA C TCTTAGGTGG-3', and *zwf*-3'-forward: 5'-GTGTGT CGACCCTCGCGCAGCGGCGCATCCG-GATGC-3'). The primers also generate internal *Not*I, *Pac*I, *Sph*I, *Sfi*I, and *Swa*I in order to facilitate cloning of DNA fragments into the  $\Delta zwf$  for stable chromosomal integration without antibiotic resistance. This vector is referred to as pCVD442- $\Delta zwf$ . The presence of the deletion, in Amp<sup>S</sup> Suc<sup>R</sup> colonies, was detected by PCR using the following primers:*zwf*-FL-forward: 5'-ATATTACTCCTGGCGACTGC-3' and *zwf*-FL-reverse: 5'-CGACAATACGCTGTGTTACG-3'. Wild type produces a 2,026 base pair product whereas the mutant produces a 608 base pair (bp) product, a difference of 1418 bp, which corresponds to the size of the *zwf* gene (1475 bp minus a 57 bp multiple cloning site that replaces the open reading frame).

#### $\beta$ -galactosidase Assay

For  $\beta$ -galactosidase expression, *lacZ* was cloned into the high copy vector pSP72 (Promega) in *E. coli*, transformed into *Salmonella* strains (via restriction defective *Salmonella* strain YS501 [31]), and screened for bright blue colonies on LB agar containing 40  $\mu$ g/ml X-gal. *lacZ* was cloned from *E. coli* K-12 MG1655 [32] obtained from the Yale *E. coli* Genetic Stock Center (New Haven, CT) by PCR using the primers BGF1 5'-GATCGGATCCATGACCATGAT-TACGGATTCACTGGC-3' and BGR1 5'-GATCAAGCTTT-TATTTTTGACACCAGACCAACTGG-3'. The PCR product was cut with *Bam*HI and *Hind*III and cloned into the plas-

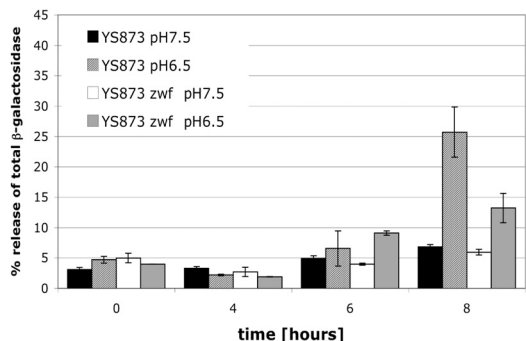


**Figure 7**  
**zwf suppresses sensitivity to acidic pH in LB broth in air, and to 5% CO<sub>2</sub> in LB broth pH 6.6, but not pH 7.6.**  
 Strains were grown in LB broth buffered to pH 6.6, or pH 7.6, in either air (A and C) or 5% CO<sub>2</sub> (B and D).

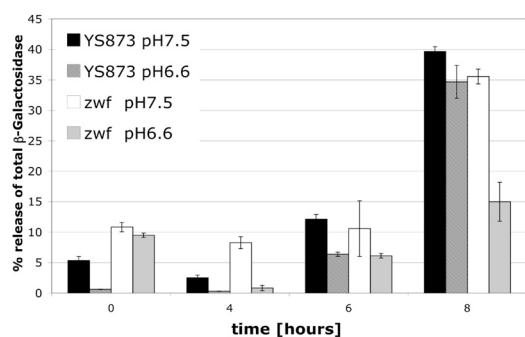
mid pSP72 (Promega, Madison, WI) which had been cut with the same enzymes, transformed into DH5α, and selected for bright blue colonies on LB-amp plates containing 40 μg/ml X-gal. The plasmid was subsequently transformed to the restriction minus methylation plus strain YS501 before transforming other *Salmonella* strains. β-gal assays were performed according to the instructions for the Galacto-Star™ chemiluminescent reporter gene assay system (Applied Biosystems, Bedford, Massachusetts). Briefly, 1 ml of bacterial culture expressing β-gal from pSP72lacZ was pelleted at 13,000 × g for 5 min. Supernatants were filtered through a 0.2 μm syringe filter and then assayed immediately or frozen at -80 °C until assayed with no further processing. Cell pellets were quickly freeze-thawed and suspended in 50 μl or 200 μl B-PER™ bacterial cell lysis reagent (Pierce Chemical) containing 10 mg/ml lysozyme (Sigma). Bacteria were allowed to lyse for 10–20 min. at room temperature and

were then placed on ice. All reagents and samples were allowed to adjust to room temperature before use. Filtered supernatants and bacterial lysates were diluted as needed in Galacto-Star™ Lysis Solution or assayed directly. β-gal standard curves were made by preparing recombinant β-gal (Sigma, 600 units/mg) to 4.3 mg/ml stock concentration in 1× PBS. The stock was diluted in Lysis Solution to prepare a standard curve of 100 ng/ml- 0.05 ng/ml in doubling dilutions. 20 μl of standard or sample was added to each well of a 96-well tissue culture plate. 100 μl of Galacto-Star™ Substrate, diluted 1:50 in Reaction Buffer Diluent, was added to each well and the plate rotated gently to mix. The plate was incubated for 90 minutes at 25 °C in the dark and then read for 1 second/well in an L-max™ plate luminometer (Molecular Devices). Sample light units/ml were compared to the standard curve and values converted to units β-gal/ml. Percent release of β-gal was determined by dividing units/ml supernatant by total

## A. $\beta$ -gal release in low pH and Air



## B. $\beta$ -gal release in low pH and CO<sub>2</sub>



**Figure 8**  
 **$\beta$ -galactosidase release assays confirm cell lysis in LB broth, pH 6.6, in air; *zwf* inhibits cell lysis in LB broth, pH 6.6, in air and in LB broth, pH 6.6, but not pH 7.6, in the presence of 5% CO<sub>2</sub>.** Release of  $\beta$ -galactosidase from the cytosol of the bacteria was used to test if the growth defects observed in YS873 and YS873 *zwf* resulted from cell lysis. Strains grown in LB broth at either pH 6.5, or pH 7.5, under either ambient air (A) or 5% CO<sub>2</sub> (B) conditions.

units/ml (units/ml supernatant + units/ml pellet). All samples were assayed in triplicate.

### Authors' contributions

DB was responsible for the overall project concept and design. VK, SRM and DB designed and planned the experiments. VK, SRM, JP, KT, MI, MK, KBL and DB performed the experiments and analyzed the results. VK, SRM, KBL and DB wrote the manuscript. All authors read and approved the final manuscript.

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