msbB deletion confers acute sensitivity to CO₂ 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 CO₂ (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 CO₂ acidifies LB broth media, buffering LB to pH 7.5 did not restore growth of msbB mutants in CO₂, indicating that the CO₂-induced growth defects are not due to the effect of CO₂ on the pH of the media. A transposon insertion in the glucose metabolism gene zwf compensates for the CO₂ sensitivity of msbB Salmonella. The msbB zwf mutants grow on agar, or in broth, in the presence of 5% CO₂. 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 CO₂, acidic pH, and high osmolarity. Disruption of zwf in msbB mutants restores growth in 5% CO₂ 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 TNFα-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 × 10⁸ CFU/m² and 1 × 10⁹/m² [6]. Toso et al. [6] noted that YS1646 (suppressed msbB strain, see below) grew best in air without added CO₂.

The potential to grow in acidic and CO₂-rich environments is a hallmark of pathogenic bacteria, enhancing persistence within phagocytes and survival inside the host. Sensitivity to CO₂ and low pH of msbB Salmonella strains might explain poor colonization of tumors, which often contain high levels of CO₂ 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% CO₂ 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% CO₂ atmosphere in LB media as well as under low pH conditions when compared to wild-type Salmonella. Furthermore, several CO₂ 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

CO₂ sensitivity of msbB Salmonella

CO₂ sensitivity was first observed when YS1646, an msbB purI Suswan deletion strain of Salmonella Typhimurium, was plated on blood or LB plates and incubated in a 5% CO₂ incubator (Caroline Clairmont, personal communication; Toso et al., 2002). Suswan 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 µl of a 10⁻⁵ dilution of a culture grown under non-selective conditions) to duplicate plates incubated at 37°C in either air or 5% CO₂, few or no colonies of YS1646 were observed after 16 hours of incubation at 37°C in 5% CO₂ (Figure 1). However, by plating more cells, the presence of a few resistant colonies could be detected, as we obtained 3.3 × 10⁸ CFU/ml on plates incubated in air and 1.7 × 10⁷ CFU/ml on plates incubated in the presence of 5% CO₂, a greater than 3 log reduction.

This CO₂ 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% CO₂ (ATCC 14028: Figure 1; other strains: data not shown). Interestingly, msbB E. coli (KL423) was not sensitive to CO₂ (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.

CO₂ sensitivity was found in all msbB Salmonella strains tested so far, indicating that CO₂ 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 CO₂ was completely restored when msbB was expressed from a plasmid (pSM21(msbBN)) (see Table 1).

The somA (for EGTA and salt resistance) and Suswan deletion (for EGTA, salt, and galactose-MacConkey resistance) msbB suppressors do NOT suppress sensitivity to 5% CO₂

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 Suswan 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 Suswan deletion in YS1646...
suppresses sensitivity to EGTA, salt, and galactose MacConkey media [9]. However, neither the somA mutation nor the Suwan deletion suppresses MsbB-mediated sensitivity to 5% CO₂ (Suwan 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% CO₂ at 37°C. Since we have not yet identified all of the genes within the Suwan deletion that are responsible for the suppressor phenotype, we focused our study on YS873, which has clearly defined mutations in msbB and somA.

**CO₂ resistant mutations are detected at high frequency in msbB somA Salmonella**

Subsequent experiments revealed that spontaneous CO₂ resistant mutants are detected when higher numbers of YS873 bacteria are plated and incubated under 5% CO₂ conditions. The mutation frequency of spontaneous CO₂ mutants from an MSB broth culture was determined to be ~3 out of 10⁴ (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 CO₂ sensitivity**

In our preliminary studies, several spontaneous CO₂ 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 CO₂ sensitivity, a random Tn5 insertion library of YS1646 was created and selected on LB agar in 5% CO₂. 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 CO₂ (Figure 2). A non-polar deletion (see materials and methods) was created in zwf (Δzwf82) 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 CO₂ 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^+ strains on agar media in both wild type and msbB genetic backgrounds.

**Gluconate prevents suppression of CO₂ 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 CO₂ gas. Perhaps a threshold of CO₂ must be passed to inhibit the growth of msbB Salmonella and a loss-of-function mutation in zwf allows for the CO₂ level to remain below this threshold. Previous reports of zwf E. coli show reduced CO₂ production when grown in minimal media with acetate or pyruvate as a carbon source. However, zwf E. coli produced more CO₂ 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                                                                 |
|--------------------------|-----------------|---------------------------------------------|--------------------------------------------------------------------------------------|
| S. enterica serovar Typhimurium |                 |                                             |                                                                                      |
| I4028                    | I4028           | Wild type                                   | ATCC 14028                                                                            |
| I4028Δzwf                | I4028           | Δzwf82                                      | Replacement of zwf gene with Δzwf82 by homologous recombination                      |
| I4028 gnd                | I4028           | gnd-189·MudJ (KanR)                         | P22 DM4483 × I4028 → Kan40R                                                          |
| YS1646(=VNP20009)        | I4028           | ΔmsbB82 ΔpurI ΔSuwwan                      | YS1646 × P22 Tn5 pool (on I4028) → selection on LB plates in 5% CO₂                   |
| YSI                       | I4028           | msh8::Stet                                 | [4]                                                                                  |
| YSI msh8+                 | YSI             | msh8::Stet/pSM21msh8* (AmpR)               | Plasmid pSM21 [4] into YSI                                                            |
| YSI zwf(=YSI1)           | YSI             | msh8::Stet zwf;Tn5 (KanR)                  | P22·VNP20057 × YS1 → Kan40R                                                          |
| YS873                    | I4028           | msh8::Stet somA1 zby/0:Tn10                | [4]                                                                                  |
| YS873 msh8+ (=YS8731)    | YS873           | msh8::Stet somA1 zby/0:Tn10 pSM21msh8* (AmpR) | Plasmid pSM21 [4] into YS873                                                        |
| YS873 zwf(=YS8732)       | YS873           | msh8::Stet somA1 zby/0:Tn10 Δzwf82         | P22·VNP20057 × YS873 → Kan40R                                                        |
| YS873 Δzwf(=YS8733)      | YS873           | msh8::Stet somA1 zby/0:Tn10 Δzwf82         | Replacement of zwf;Tn5 gene in YS873zwf with Δzwf82 by homologous recombination     |
| YS873 gnd(=YS8734)       | YS873           | msh8::Stet somA1 zby/0:Tn10 gnd-189·Mudj (KanR) | P22 DM4483 × YS873 → Kan40R                                                          |
| LT2                      | LT2             | Wild type                                   | ATCC I5277                                                                            |
| DM4483                   | LT2             | gnd-189·Mudj (KanR)                         | Gift of Diana Downs and Eugene I. Vivas, U. of Wisconsin                             |
| Y5501                    | LT2             | recD541::Tn(0::Cam hsdSA29 hsdSB121 hsdR6 metA22 mmtES1 tptCZ α-452 H1-b H2-e,n,x flb-66 netN(-) rpsL120 xyl-404 galE719 | [5] |
| Salmonella enterica serovar Typhi |               |                                             |                                                                                      |
| C5029                    |                 |                                             |                                                                                      |
| Salmonella enterica serovar Typhi |               |                                             |                                                                                      |
| ATCC 33458               |                 |                                             |                                                                                      |
| E. coli K-12 MG1655       | MG1655          | F- l- rph-l                                | [32]                                                                                 |
| KL423                    | MG1655          | F- l- rph-1 msbB::ΩCm                      | [4]                                                                                  |
| pCVD442                  |                 | AmpR                                       | [10]                                                                                 |
| pCVD442Δzwf82            |                 | AmpR                                       | This study                                                                            |
| pSP72                    |                 | AmpR                                       | Promega Corporation                                                                  |
| pSP72lacZ                |                 | lacZ, AmpR                                 | This study                                                                            |
| pSM21                    |                 | msh8, AmpR                                 | [4]                                                                                  |
studies will be required to clarify the production of CO₂
by Salmonella grown in Luria-Bertani-based media and its
contribution to CO₂ 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 CO₂ sensi-
tivity in our mutants. Growth of YS873 zwf was tested on
LB-0 plates containing 0.33% gluconate in ambient air
and 5% CO₂ (Figures 3I and 3J). As we hypothesized,
YS873 zwf was not able to grow on LB-0 gluconate in 5%
CO₂. Thus, we confirmed that the zwf’s suppression of
CO₂ 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 down-
stream PPP metabolite is responsible for mediating CO₂
resistance, we tested for CO₂ 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 CO₂ sensitive (data not shown). Therefore, we
conclude that the production of 6-phosphogluconate, by
either Zwf or gluconate kinase, contributes to CO₂ sensi-
tivity in an msbB genetic background.

zwf mutation suppresses both msbB-induced CO₂
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 com-
pletely inhibited when the plates are incubated in the
presence of 5% CO₂. The introduction of the zwf mutation
completely compensates for the phenotype and allows the
bacteria to grow under 5% CO₂ on all three media (Fig-
ures 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. Interest-
ingly, 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-
duced 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 phos-
phates of lipid A can help to compensate for the lack of
the myristic acid residue, we investigated whether Mg²⁺
and Ca²⁺ would protect against the detrimental effects of
5% CO₂. On agar plates, Mg²⁺ and Ca²⁺ showed partial
protection in YS873 (Figure 3D).

YS873, which contains the EGTA and salt resistance sup-
pressor mutation somA [4], grows well on LB (Figure 3A),
MSB (Figure 3C), LB-0 (Figure 3E) and LB-0 sucrose (Fig-
ure 3G) agar plates in air, but not when the plates are incu-
bated in 5% CO₂ (Figures 3B, 3D, 3F, and 3H). In
contrast, the strain YS873 zwf is able to grow on all of
these media in CO₂, indicating that the zwf mutation can
compensate for the growth defect of msbB strains in CO₂
(Figure 3). Subsequent experiments were performed using
the YS873 (msbB somA) genetic background because
unsuppressed msbB Salmonella can not grow under mam-
malian physiological salt conditions [4].

msbB somA Salmonella are sensitive to CO₂ 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 CO₂.
incubated in the presence or absence of 5% CO₂. 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% CO₂. A significant decrease in CFU is observed (Figure 4A), indicating that YS873 cells lose viability in the presence of 5% CO₂ 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% CO₂ are attenuated in comparison to those observed in LB broth. There is no decrease in viability in YS873 in LB-0 in 5% CO₂, although there is impaired growth in both YS873 and YS873 zwf in LB-0 in the presence of CO₂ compared to growth in the absence of CO₂ (Figure 4B).

YS873 has severe morphological defects in LB broth under 5% CO₂ 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% CO₂ (Figure 4), we examined msbB mutants grown in the presence of 5% CO₂ to determine if there are any defects in cell morphology or chromosome segregation. Differential interference contrast (DIC) microscopy shows striking morphological defects under CO₂ 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).

β-galactosidase assays confirm cell lysis in LB in the presence of 5% CO₂

As shown in Figures 5O and 5P, zwf suppresses the severe morphological defects in YS873 grown in LB in the presence of 5% CO₂. Many cells are elongated but lack gross morphological defects. Growth in LB in a 5% CO₂ 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 CO₂ (20/95 cells). Thus, we conclude that both CO₂ and Zwf can, either directly or indirectly, affect cell division.

CO₂ 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% CO₂. zwf mutation suppresses both msbB-induced CO₂ 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% CO₂; C, MSB media in air; D, MSB media in 5% CO₂; E, LB-0 media in air; F, LB-0 media in 5% CO₂; G, LB-0 media containing sucrose (total 455 milliosmoles) in air; H, LB-0 media containing sucrose in 5% CO₂; I, LB-0 + gluconate (glucon.) in air; J, LB-0 + gluconate in 5% CO₂.
CO₂. Since CO₂ can acidify bicarbonate buffered media, we tested whether part of the CO₂ sensitivity was due to acidification of the media. Thus, to test if increased or decreased pH would alter sensitivity to CO₂ 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% CO₂. 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% CO₂. 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% CO₂ inhibited the growth of YS873 and YS873 zwf in LB pH 6.6 and 7.6 (Figure 7B). Although zwf protects against 5% CO₂ in LB broth pH 6.6 (Fig 7B), it does not significantly improve survival in the presence of 5% CO₂ 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% CO₂.

**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-
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% CO₂ 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% CO₂ 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% CO₂, 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% CO₂ at pH 6.6 or 7.6, and in YS873 zwf grown in the presence of 5% CO₂ in LB pH 7.5. YS873 zwf exhibited significantly less lysis in the presence of 5% CO₂ in LB broth pH 6.6, showing that a loss-of-function mutation in zwf significantly suppresses sensitivity to CO₂ 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% CO₂ growth conditions. Significantly, msbB Salmonella are sensitive to the conditions found within mammals, where blood has significant levels of salt and CO₂; we therefore screened for a suppressor of msbB-associated CO₂ sensitivity.

**zwf suppresses CO₂ 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 fimC, 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% CO₂ and pH 6.5 conditions, suggesting that the expression of zwf is detrimental for growth of msbB mutants in an acidic or increased CO₂ 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 CO₂ suggests that the production of 6-phosphogluconate, by either Zwf or glucuronate kinase, contributes to CO₂ 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% CO₂ conditions, the lack of virulence may be partially due to the inability of msbB Salmonella to grow in the presence of the 5% CO₂. 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% CO₂. 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 CO₂ 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% CO₂ 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 CO₂ 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 CO₂ and ambient air. We have
created an msbB somA zwf Salmonella strain that is resistant to growth under acidic or 5% CO₂ conditions. This strain contains a loss-of-function mutation in zwf, an enzyme in the pentose phosphate pathway that produces CO₂ 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% CO₂, 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/int201 (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 MgSO₄ and 2 mM CaCl₂. LB-0 is LB media with no NaCl. Buffered LB pH 7.5 and pH 6.5 consisted of LB-0 with 100 mM NaPO₄ 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* genotype**
In order to confirm that the observed CO₂ 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% CO₂. O.D₅₆₀ 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 Axioscope 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.
**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. 10-6 and 10-4 dilutions were plated in duplicates onto LB agar and incubated in air and CO2 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 μFD for transformation of YS1 and 1.7 kV, 186 ohms and 25 μFD for transformation of 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 (KanR) clones of YS1646 were pooled. The pool was screened for CO2 resistance by plating dilutions to low selection for CO2 resistance by plating dilutions to petridishes containing various pools of amino acids and bases [30]. Following selection for CO2 resistance by plating dilutions to LB-Kan and incubating in 5% CO2, 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-CO2 conditions in order to separate spontaneous mutants from Tn5-based suppressors. Transposon-associated Tn5 insertions were identified by replica plating in air and CO2. Mapping of the insertion sites was performed by using the GenomeWalker™ kit (Clonetech, 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-forward: 5’-GTGTGATCGTCGCTGTCGTCCGCCGCCAGCGGCGCATCCG-3' (with added Sall), zwf-3'-forward: 5’-GTGTGATCGTCGCTGTCGTCCGCCGCCAGCGGCGCATCCG-3' (with added Sphi), zwf-3'-reverse: 5’-GTGTGATCGTCGCTGTCGTCCGCCGCCAGCGGCGCATCCG-3'. The primers also generate internal NolI, Paci, Sphi, Sfl, and Swal in order to facilitate cloning of DNA fragments into the zwf region for stable chromosomal integration without antibiotic resistance. This vector is referred to as pCVD442-Azwf. The presence of the deletion, in Amp<sup>®</sup> Suc<sup>®</sup> colonies, was detected by PCR using the following primers: zwf-FL-forward: 5’-ATATTACCTCCGCGGAGCTTG-3' and zwf-FL-reverse: 5’-CGAACAATACGCTGTGTTACG-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).

**β-galactosidase Assay**

For β-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 μg/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’-GATCCGATCCATGACCATGAT-TACGATTCGACGCGCCGG-3' and BGR1 5’-GATCAAGCTT-TATTCTTGACACAGACCATGCGG-3'. The PCR product was cut with BamHI and HindIII and cloned into the plas-
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 BPER® 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/ml supernatant by total

**Figure 7**

*zwf* suppresses sensitivity to acidic pH in LB broth in air, and to 5% CO₂ 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% CO₂ (B and D).
A. \( \beta \)-gal release in low pH and Air

between 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% \( \mathrm{CO}_2 \) (B) conditions.

B. \( \beta \)-gal release in low pH and \( \mathrm{CO}_2 \)

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% \( \mathrm{CO}_2 \). 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% \( \mathrm{CO}_2 \) (B) conditions.

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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