Supplemental Material for:

Cell wall inhibition in L-forms or via β-lactam antibiotics induces reactive oxygen-mediated bacterial killing through increased glycolytic flux

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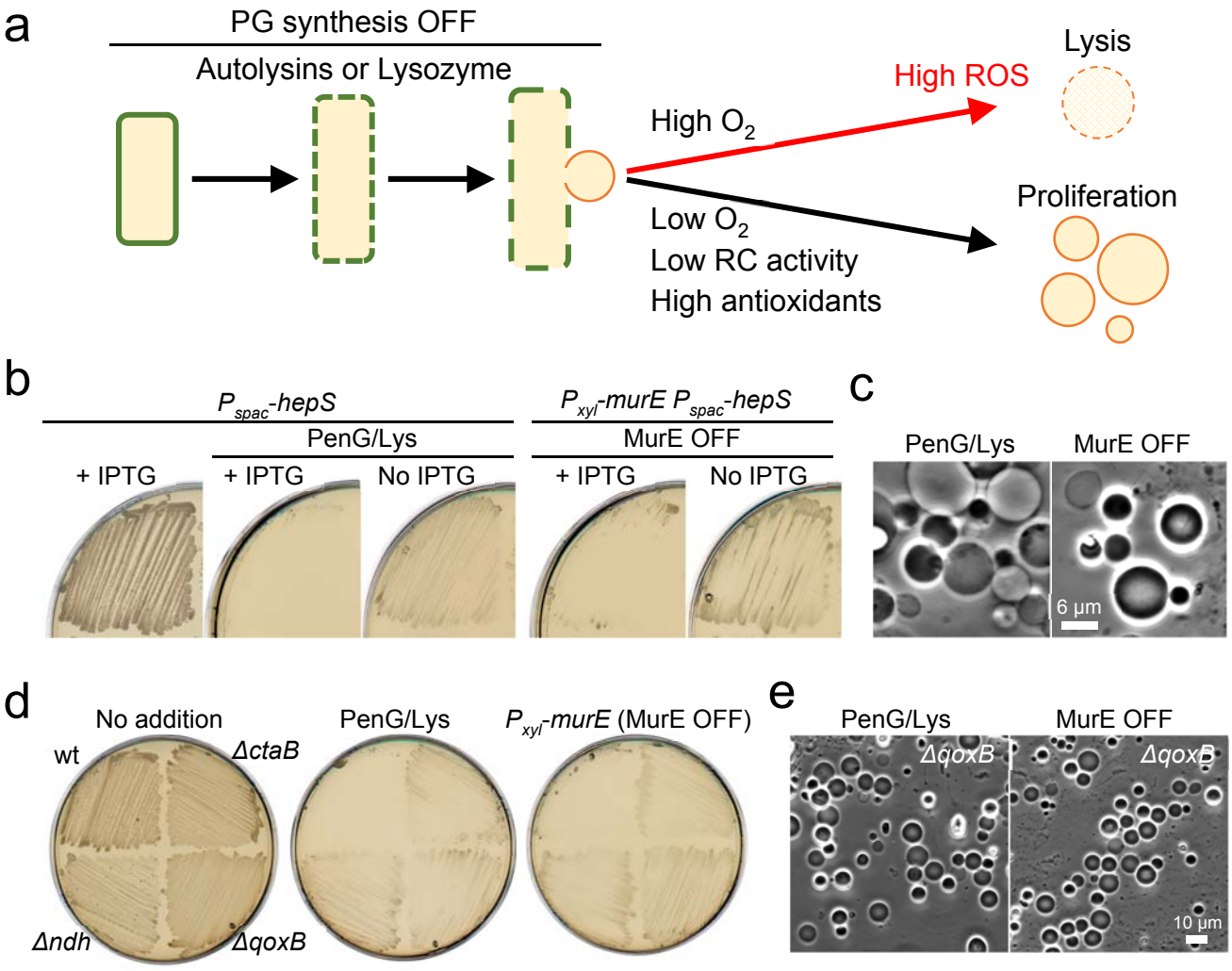
Contents:


a
PG synthesis OFF
Autolysins or Lysozyme
High O_2
High ROS
Lysis
Low O_2
Low RC activity
High antioxidants
Proliferation
b
P_{spac}-hepS
PenG/Lys
+ IPTG
+ IPTG
No IPTG
P_{xyl}-murE P_{spac}-hepS
MurE OFF
+ IPTG
No IPTG
c
PenG/Lys
MurE OFF
6 μm
d
No addition
PenG/Lys
P_{xyl}-murE (MurE OFF)
wt
ΔctaB
Δndh
ΔqoxB
e
PenG/Lys
MurE OFF
ΔqoxB
ΔqoxB
10 μm

Supplementary Figure 1. (Related to Figures 1 and 3) Effects of respiratory chain on L-form growth

a, Schematic representation of L-form switch by inhibiting PG precursor pathway in *B*. *subtilis*. The increase of cellular ROS levels in the wall deficient cells results in cell death and counteraction of the ROS production promotes L-form proliferation.

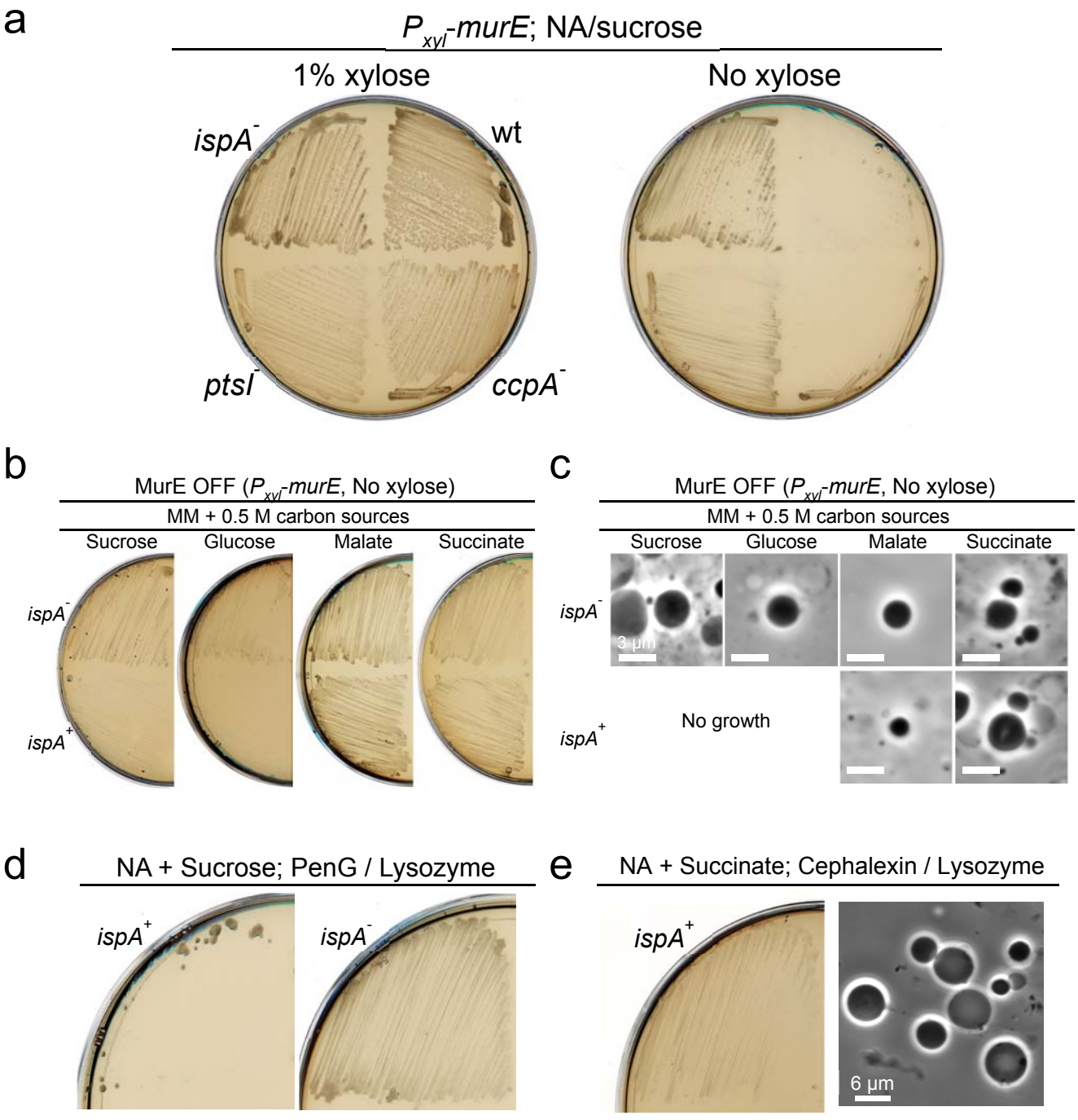
b, *B*. *subtilis* strains YK1450 (P_{spac}-*hepS*) and YK1454 (P_{xyl}-*murE* P_{spac}-*hepS*) were streaked on NA/sucrose plates with or without 1 mM IPTG in the absence of xylose, and incubated at 30°C.

c, Phase contrast micrographs of *B*. *subtilis* L-forms were taken from the plates shown in panel **b**.

d, *B*. *subtilis* strains 168CA (wild-type), YK1460 (*ctaB*::*Tn*), YK1461 (*ndh*::*Tn*) and YK1462 (*qoxB*::*Tn*) were streaked on NA/sucrose plates with (middle) or without (left) 200 μg/ml PenG and 100 μg/ml chicken egg white lysozyme, and incubated at 30°C. *B*. *subtilis* strains BS115 (P_{xyl}-*murE*), YK1816 (P_{xyl}-*murE ndh*::*Tn*), YK1817 (P_{xyl}-*murE qoxB*::*Tn*) and YK1818 (P_{xyl}-*murE ctaB*::*Tn*) were streaked on NA/sucrose plates (no xylose), and incubated at 30°C (right).

e, Phase contrast micrographs of *B*. *subtilis* L-forms with a *qoxB* mutation (YK1817) were taken from the plates shown in panel **d**.

The figures are representative of at least three independent experiments (**b**-**e**).


a
P_{xyl}-*murE*; NA/sucrose
1% xylose
No xylose
ispA$^-$
wt
ptsI$^-$
ccpA$^-$
b
MurE OFF (P_{xyl}-*murE*, No xylose)
MM + 0.5 M carbon sources
Sucrose
Glucose
Malate
Succinate
ispA$^-$
ispA$^+$
c
MurE OFF (P_{xyl}-*murE*, No xylose)
MM + 0.5 M carbon sources
Sucrose
Glucose
Malate
Succinate
ispA$^-$
3 µm
ispA$^+$
No growth
d
NA + Sucrose; PenG / Lysozyme
ispA$^+$
ispA$^-$
e
NA + Succinate; Cephalexin / Lysozyme
ispA$^+$
6 µm

Supplementary Figure 2. (Related to Figures 2 and 3) Contrasting effects of carbon sources on L-form growth

a, *B*. *subtilis* strains BS115 (P_{xyl}-*murE*), LR2 (P_{xyl}-*murE ispA*$^-$), YK1391 (P_{xyl}-*murE ccpA*::*tn*) and YK1392 (P_{xyl}-*murE ptsI*::*tn*) were streaked on NA/sucrose plates with or without 1% xylose, and incubated at 30°C.

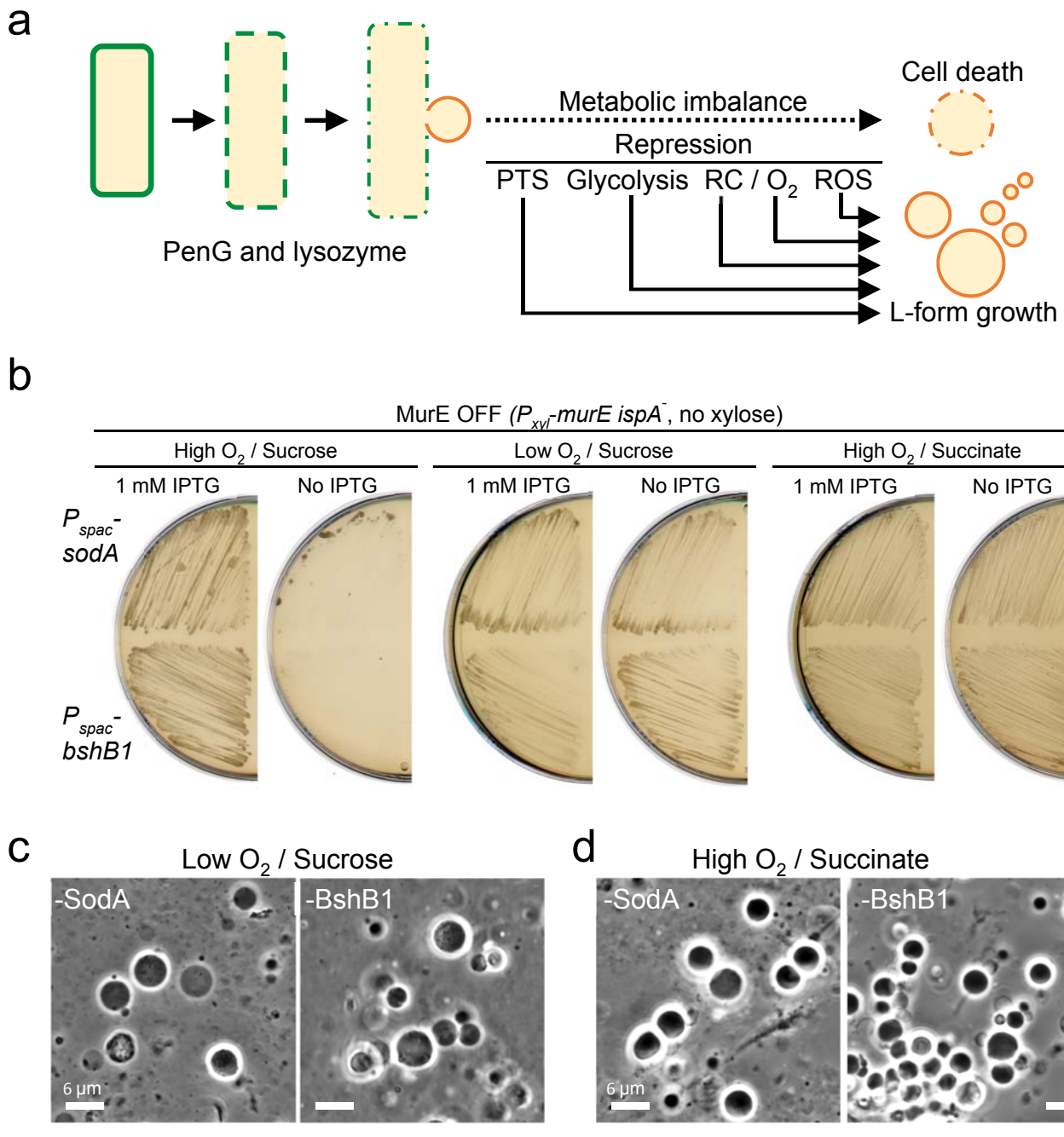
b, Effects of carbon sources on L-form growth on minimal medium (MM). *B*. *subtilis* strains BS115 (P_{xyl}-*murE ispA*$^+$) and LR2 (P_{xyl}-*murE ispA*$^-$) were streaked on MM plates with 0.5 M various carbon sources (sucrose, glucose, malate or succinate) in the absence of xylose (MurE OFF), and incubated at 30°C.

c, Phase contrast micrographs of BS115 or LR2 were taken from the plates shown in panel **b**.

d, Effects of an *ispA* mutation on *B*. *subtilis* L-form growth on sucrose plates containing PenG and lysozyme. *B*. *subtilis* wild-type strain (168CA; *murE*$^+$ *ispA*$^+$) and RM81 (*murE*$^+$ *ispA*$^-$) were streaked on NA/sucrose plates containing 200 μg/ml PenG and 100 μg/ml chicken egg white lysozyme, and incubated at 30°C for 2-3 days.

e, Effects of succinate on *B*. *subtilis* L-form growth. *B*. *subtilis* wild-type strain (168CA) was streaked on NA/succinate plates containing 100 μg/ml cephalexin and 100 μg/ml lysozyme, and incubated at 30°C. Phase contrast micrograph of *B*. *subtilis* L-forms was taken from the plate (left).

The figures are representative of at least three independent experiments (**a**-**e**).


a
PenG and lysozyme
Metabolic imbalance
Repression
PTS
Glycolysis
RC / O2
ROS
Cell death
L-form growth
b
MurE OFF (PxyI-murE ispA-, no xylose)
High O2 / Sucrose
Low O2 / Sucrose
High O2 / Succinate
1 mM IPTG
No IPTG
Pspac-sodA
Pspac-bshB1
c
Low O2 / Sucrose
-SodA
-BshB1
6 µm
d
High O2 / Succinate
-SodA
-BshB1
6 µm

Supplementary Figure 3. (Related to Figures 3 and 4) Oxidative stress does not prevent L-form growth under gluconeogenic conditions.

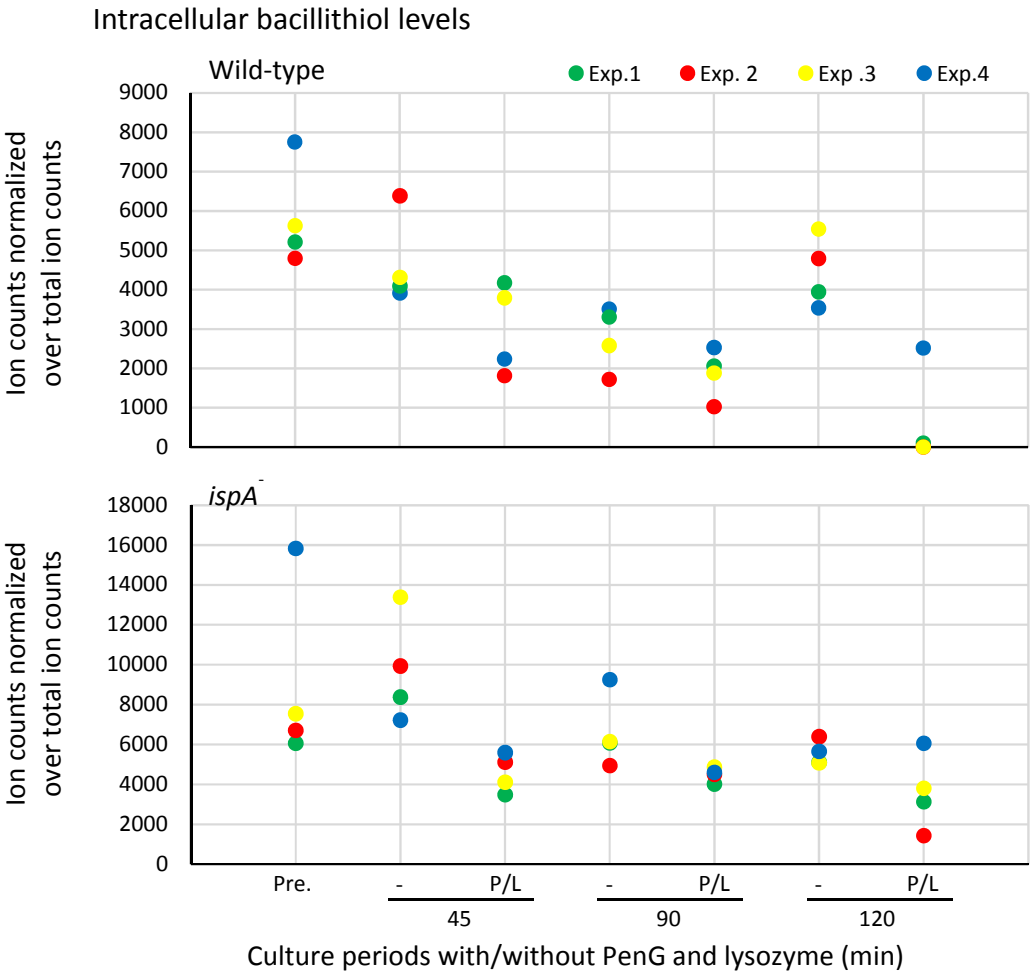
a, Schematic representation of L-form death/growth phenotype in *B*. *subtilis* under glycolytic conditions.

b, Effects of oxidative stress on L-form switch and growth under various culture conditions. *B*. *subtilis* strains YK1604 (*P_{xyl}-murE ispA$^-$ P_{spac}-bshB1*) and YK2028 (*P_{xyl}-murE ispA$^-$ P_{spac}-sodA*) were streaked on NA/sucrose or succinate plates with or without IPTG in the absence of xylose (MurE OFF), and incubated at 30°C under aerobic (High O_2) or anaerobic (Low O_2) conditions.

c, **d**, Phase contrast micrographs of L-forms were taken from the NA/sucrose (**c**; No IPTG, Low O_2 / Sucrose) or NA/succinate plate (**d**; No IPTG, High O_2 / Succinate) shown in panel **b**.

The figures are representative of at least two independent experiments (**b**-**d**).

We previously showed that the expression levels of many genes required for defence systems against oxidative stress are higher in *B*. *subtilis* L-forms than those of parental walled cells in NA/sucrose medium, and also that the several antioxidant systems, such as superoxide dismutase (*sodA*) and bacillithiol synthesis (*bshB1*), are essential for L-form growth[1] (**b**, High O_2 / Sucrose). As anticipated, it turned out that these systems are no longer required for L-form growth under anaerobic conditions (**b**, **c**, Low O_2 / Sucrose), consistent with the notion that the ROS originate from aerobic respiration. We then examined the possible role of the antioxidant systems on L-form growth in the presence of succinate under aerobic conditions and found that they are not required (**b**, **d**, High O_2 / Succinate). This suggests that the abnormal increase of ROS in the L-form transition is largely suppressed under gluconeogenic conditions.


Intracellular bacillithiol levels
Wild-type
Exp.1
Exp. 2
Exp .3
Exp.4
Ion counts normalized over total ion counts
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
ispA-
Ion counts normalized over total ion counts
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
Pre.
-
P/L
-
P/L
-
P/L
45
90
120
Culture periods with/without PenG and lysozyme (min)

Supplementary Figure 4. (Related to Figure 4) Effects of the L-form transition on intracellular bacillithiol levels.

The dot plots show the total pool size of intracellular bacillithiol from ~10^8 cells of *B. subtilis* wild-type (168CA) and *ispA*$^-$ (RM81) cultures on NA/glucose plates with PenG (P) and lysozyme (L). Pre-cultures of *B. subtilis*, before incubation in the presence of labelled glucose, were also analysed (Pre.). The identification of bacillithiol is based on m/z ion counts, which are normalised over total ion counts. The each dots represent the averages from four biological replicates (Exp. 1-4).

Supplementary Video 1. (Related to Figure 3) Contrasting effects of glucose and succinate on L-form death/growth phenotype

B. subtilis wild-type cells (strain 168CA) were imaged in NB/glucose (left) or succinate (right) with 0.2% agar in the presence of PenG and lysozyme. PC images were acquired every 5 min (Numbers in the top right corner). The movie displayed at 5 frames per second. Scale bar represents 5 μm. The figures are representative of at least two independent experiments.

Supplementary Table 1. (Related to Figure 4) Secreted succinate levels under glycolytic conditions in the presence or absence of PenG and lysozyme

	μM/total ions counts*10^9 (standard deviation)[a]			
	Wild-type (strain 168CA)		*ispA*$^-$ (strain RM81)	
Time (min)	No addition	PenG and lysozyme	No addition	PenG and lysozyme
0 (pre-culture)	0.5795 (0.0259)	-	0.8079 (0.0413)	-
45	0.6531 (0.0353)	0.2810 (0.0103)	0.8211 (0.0567)	0.2975 (0.0076)
90	0.6218 (0.0455)	0.2446 (0.0129)	0.7878 (0.0563)	0.2921 (0.0093)
120	0.6316 (0.0273)	0.2283 (0.0128)	0.7587 (0.0500)	0.2638 (0.0148)

[a]The averages and the standard deviation of succinate concentrations in ~10^8 cells of wild-type or *ispA* mutant from at least three biological replicates were shown. The samples were identical used for extracellular pyruvate analysis (Fig. 4B)

Supplementary Table 2. (Related to Figure 5) Total viable *S. aureus* cells counts (CFU/ml) under various growth conditions

	Experiment 1[a]		Experiment 2	
Time after addition of PenG (hr)	NB/Glucose	NB/Succinate	NB/Glucose	NB/Succinate
0	1.8 x 10^8	1.7 x 10^8	3.2 x 10^8	2.2 x 10^8
1	9.8 x 10^7	1.1 x 10^8	1.8 x 10^8	1.8 x 10^8
3	6.5 x 10^7	1.1 x 10^8	9.2 x 10^7	1.6 x 10^8
5	3.4 x 10^7	1.1 x 10^8	2.6 x 10^7	6.2 x 10^7
24	7.0 x 10^4	7.1 x 10^7	5.0 x 10^5	4.0 x 10^7

[a]The data from Experiment 1 was used to generate figure 5E.

Supplementary Table 3. Bacterial strains, oligonucleotides and plasmids

Bacterial strains	
Bacillus subtilis	
168CA; *trpC2* (wild-type)	Lab. stock
RM81; 168CA *xseB*::*Tn kan* (*ispA*$^-$)	[2]
BS115; 168CA *ΩspoVD*::*cat* P_{xyl}-*murE ΩamyE*::*(tet xylR)*	[3]
LR2; BS115 *xseB** (Frameshift 22T>-) (*ispA*$^-$)	[2]
BS116; 168CA *ΩspoVD*::*spc* P_{xyl}-*murE ΩamyE*::*(tet xylR)*	Lab. stock
YK1450; 168CA *ΩhepS*::pMutin4-*erm* P_{spac}-*hepS*	{Kawai 2015}
YK1454; BS116 *ΩhepS*::pMutin4-*erm* P_{spac}-*hepS*	{Kawai 2015}
YK1391; BS115 *ccpA*::*Tn kan*	[4]
YK1392; BS115 *ptsI*::*Tn kan*	[4]
YK1460; 168CA *ctaB*::*Tn kan*	{Kawai 2015}
YK1461; 168CA *ndh*::*Tn kan*	{Kawai 2015}
YK1462; 168CA *qoxB*::*Tn kan*	{Kawai 2015}
YK1563; LR2 *ΩglmM*::pMutin4-*erm* P_{spac}-*glmM*	This work
YK1571; BS115 *ΩgapA*::pMutin4-*erm* P_{spac}-*gapA*	This work
YK1601; BS115 *ΩptsHI*::pMutin4-*erm* P_{spac}-*ptsHI*	This work
YK1602; BS115 *ΩptsG*::pMutin4-*erm ΔptsG*	This work
YK1604; LR2 *ΩbshB1*::pMutin4-*erm* P_{spac}-*bshB1*	[1]
YK1621; BS116 *ΩgapA*::pMutin4-*erm* P_{spac}-*gapA amyE*::P_{spacHY}-*gapA cat*	This work
YK1763; YK1563 *ndh*::*Tn kan*	{Kawai 2015}
YK1764; YK1563 *qoxB*::*Tn kan*	{Kawai 2015}
YK1765; YK1563 *ctaB*::*Tn kan*	{Kawai 2015}
YK1816; BS115 *ndh*::*Tn kan*	{Kawai 2015}
YK1817; BS115 *qoxB*::*Tn kan*	{Kawai 2015}
YK1818; BS115 *ctaB*::*Tn kan*	{Kawai 2015}
YK1995; YK1563 *mhqR*::*Tn kan*	{Kawai 2015}
YK2028; LR2 *ΩsodA*::pMutin4-*erm* P_{spac}-*sodA*	[1]
YK2124; BS115 *ΩsacP*::pMutin4-*erm ΔsacP*	This work
YK2125; BS115 *ΩfruA*::pMutin4-*erm ΔfruA*	This work
YK2126; BS115 *ΩlevD*::pMutin4-*erm ΔlevD*	This work
Listeria monocytogenes	
EDGe	[5]
EDGe Δ*oatA*Δ*pgdA*	[5]
Staphylococcus aureus	
RN4220	[6]
Enterococcus faecium	
ATCC19434	Lab. stock
Oligonucleotides	
pM4SD-gapA-F; GGGGAATTCTCTCTCACTTATTTAAAGGAG	This work
pM4-gapA-R; GGGGGATCCAACGCCTTGTTTGCCCCAG	This work
pPL82-gapA-F; TCTTCTAGATCTCTCACTTATTTAAAGG	This work
pPL82-gapA-R; GCAGCATGCTCGAAAGAACCAAGTCAGG	This work
pM4SD-ptsHI-F; GAAGAATTCAAGCTTTAAGTTAAAAGGAG	This work
pM4-ptsHI-R; GGAGGATCCTTAGCGATACCTAAAGAC	This work
pM4-ptsG-F; GAAGAATTC GGTCCTGCATTTCTTGAG	This work
pM4-ptsG-R; GGAGGATCCATAATACCGCCGAACACC	This work
pM4-sacP-F; GAAGAATTCTTATCAGCGCGGCTCATTG	This work

pM4-sacP-R; GGAGGATCCTTAATAGGCCGCTGGCTAC	This work
pM4-fruA-F; GAAGAATTCGTCACTGTTCTTGATAAGG	This work
pM4-fruA-R; GGAGGATCCTTCAGACTCAGCTTCAAGG	This work
pM4-levD-F; GAAGAATTCATTATCAGCGGTCATGGAG	This work
pM4-levD-R; GGAGGATCCAAGCTGCCGCGTTATATGG	This work
pM4SD-glmM-F; GGGGAATTCAAAGGAGCGATTATAAAATGGG	This work
pM4-glmM-R; GGGGGATCCCCGCCTCTGCATCCATCG	This work
Plasmids	
pMutin4	[7]
pM4-P_{spac}-*gapA*	This work
pM4-P_{spac}-*ptsHI*	This work
pM4-*ΔptsG*	This work
pM4-*ΔsacP*	This work
pM4-*ΔfruA*	This work
pM4-*ΔlevD*	This work
pM4-P_{spac}-*glmM*	This work
pPL82	[8]
pPL82-P_{spacHY}-*gapA*	This work

Supplementary References

1 Kawai, Y. *et al.* Cell growth of wall-free L-form bacteria is limited by oxidative damage. *Curr. Biol.* **25**, 1613-1618, doi:10.1016/j.cub.2015.04.031 (2015).

2 Mercier, R., Kawai, Y. & Errington, J. Excess membrane synthesis drives a primitive mode of cell proliferation. *Cell* **152**, 997-1007, doi:10.1016/j.cell.2013.01.043 (2013).

3 Leaver, M., Dominguez-Cuevas, P., Coxhead, J. M., Daniel, R. A. & Errington, J. Life without a wall or division machine in *Bacillus subtilis*. *Nature* **457**, 849-853, doi:10.1038/nature07742 (2009).

4 Kawai, Y., Daniel, R. A. & Errington, J. Regulation of cell wall morphogenesis in Bacillus subtilis by recruitment of PBP1 to the MreB helix. *Mol. Microbiol.* **71**, 1131-1144, doi:10.1111/j.1365-2958.2009.06601.x (2009).

5 Aubry, C. *et al.* OatA, a peptidoglycan O-acetyltransferase involved in *Listeria monocytogenes* immune escape, is critical for virulence. *J. Infect. Dis.* **204**, 731-740, doi:10.1093/infdis/jir396 (2011).

6 Kreiswirth, B. N. *et al.* The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. *Nature* **305**, 709-712 (1983).

7 Vagner, V., Dervyn, E. & Ehrlich, S. D. A vector for systematic gene inactivation in *Bacillus subtilis*. *Microbiology* **144 (Pt 11)**, 3097-3104, doi:10.1099/00221287-144-11-3097 (1998).

8 Quisel, J. D., Burkholder, W. F. & Grossman, A. D. In vivo effects of sporulation kinases on mutant Spo0A proteins in *Bacillus subtilis*. *J. Bacteriol.* **183**, 6573-6578, doi:10.1128/JB.183.22.6573-6578.2001 (2001).