Is the expression of catalase by *Staphylococcus aureus* protective or detrimental to the survival of the bacteria when exposed to H_2O_2 in broth or after phagocytosis by monocytes? Are we disproving a dogma or misinterpreting data?

Paul M. Tulkens

based on work made in collaboration with Sandrine Lemaire,[1] Wafi Siala,[1] Roland Leclercq,[2] Barbara C. Kahl,[3] and Françoise Van Bambeke,[1]

[1] Pharmacologie cellulaire et moléculaire,
Louvain Drug Research Institute,
Université catholique de Louvain, Brussels, Belgium

[2] Service de Microbiologie,
Université de Caen Basse-Normandie, Caen, France;

[3] Institüt fur Medizinische Mikrobiologie,
Universitaetsklinikum Münster, Münster, Germany.

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Content of the presentation

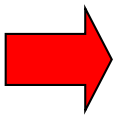
- What is the "accepted" role of catalase in *S. aureus*
- What about low or catalase-negative natural mutants ?
- What did we find ?
- What could we not do ?
- What are our current hypotheses ?

as you can see, many " ? "

The accepted role of catalase in *S. aureus*…

- *Staphylococcus aureus* invades eukaryotic cells, which shelters it from immune defences and reduces its susceptibility to most antibiotics…
  - ➢ Intracellular survival may contribute to the persistent and relapsing character of many staphylococcal infections
- In eucaryotic cells, however, *S. aureus* becomes exposed to reactive oxygen species (ROS) generated by the respiratory burst…
  - ➢ Thus, it is generally assumed that the expression of catalase by *S. aureus* will protect it and favour its intracellular survival

This concept is essentially based on original observations that high-catalase producing strains are killed relatively poorly by PMNs…



Catalase, Superoxide Dismutase, and Virulence of Staphylococcus Aureus

IN VITRO AND IN VIVO STUDIES WITH EMPHASIS ON STAPHYLOCOCCAL—LEUKOCYTE INTERACTION

GERALD L. MANDELL

From the Division of Infectious Diseases, Department of Internal Medicine, University of Virginia School of Medicine, Charlottesville, Virginia 22901

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However, absence or low activity of catalase is not always detrimental …

- A double catalase- and β-toxin negative mutant survives more readily in murine macrophages *in vitro* than its wild-type counterparts [1].
- *S. aureus* with a low catalase activity resist the bactericidal activity of leucocytes [2].
- A SCV (menadione-dependent phenotype) that survives intracellularly has a significantly diminished *katA* expression compared to its isogenic parental strain [3].
- *S. aureus* decreases its expression of *katA* immediately after invasion and during early survival in lung epithelial cells [4].

1. Martinez-Pulgarin S, Dominguez-Bernal G, Orden JA, de la FR (2009) Simultaneous lack of catalase and beta-toxin in Staphylococcus aureus leads to increased intracellular survival in macrophages and epithelial cells and to attenuated virulence in murine and ovine models. Microbiology 155: 1505-1515.
2. Nishihara S, Seki K, Masuda S (1985) Resistance of a mutant with an extremely low catalase production from Staphylococcus aureus Cowan-I strain to the bactericidal activity of human leukocytes. Microbiol Immunol 29: 151-155.
3. Kriegeskorte A, Konig S, Sander G, Pirkl A, Mahabir E, Proctor RA, von Eiff C, Peters G, Becker K (2011) Small colony variants of Staphylococcus aureus reveal distinct protein profiles. Proteomics 11: 2476-2490.
4. Garzoni C, Francois P, Huyghe A, Couzinet S, Tapparel C, Charbonnier Y, Renzoni A, Lucchini S, Lew DP, Vaudaux P, Kelley WL, Schrenzel J (2007) global view of Staphylococcus aureus whole genome expression upon internalization in human epithelial cells. BMC Genomics 8: 171

Strains used in the 1st part of our study

Parameter	strain no.				
	ATCC 25923	**2008-S080**	**2008-S419**	**UCN61**	**SH1000**
Catalase activity (U/mg protein)	**1.28 ± 0.15**	0.38 ± 0.13 *****	0.11 ± 0.06 *****	**0.0015 ± 0.003 ***	0.67 ± 0.25 *****
Susceptibility to cumene hydroperoxide (mm) [a]	**20.2 ± 0.4**	15.5 ± 0.5 *****	16.3 ± 1.2 *****	**19.7 ± 0.6**	13.3 ± 0.1 *****
Susceptibility to paraquat (mm) [a]	**29.8 ± 1.9**	ND	ND	**28.3 ± 1.6**	9.7 ± 0.5 *
Staphyloxanthin production [b]	+	+	+	**+**	+++

[a] diameter of growth inhibition zone around disks impregnated with 100 mM cumene hydroperoxide or 500 mM paraquat.
***** significantly different from the corresponding value of strain ATCC 25923 (one way ANOVA with Dunnett multiple comparisons test; P< 0.01)
[b] visual inspection
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S. aureus subs. _aureus_ strain isolated from a patient suffering from an arterial leg ulcer.
T172C and G636A mutations in _katA_
→ His-58-Tyr and Arg-212-His substitutions.
(Piau et al., 2008: Catalase-negative Staphylococcus aureus strain with point mutations in the *katA* gene. J. Clin. Microbiol. *46*, 2060-2061).

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This strain produces almost no catalase

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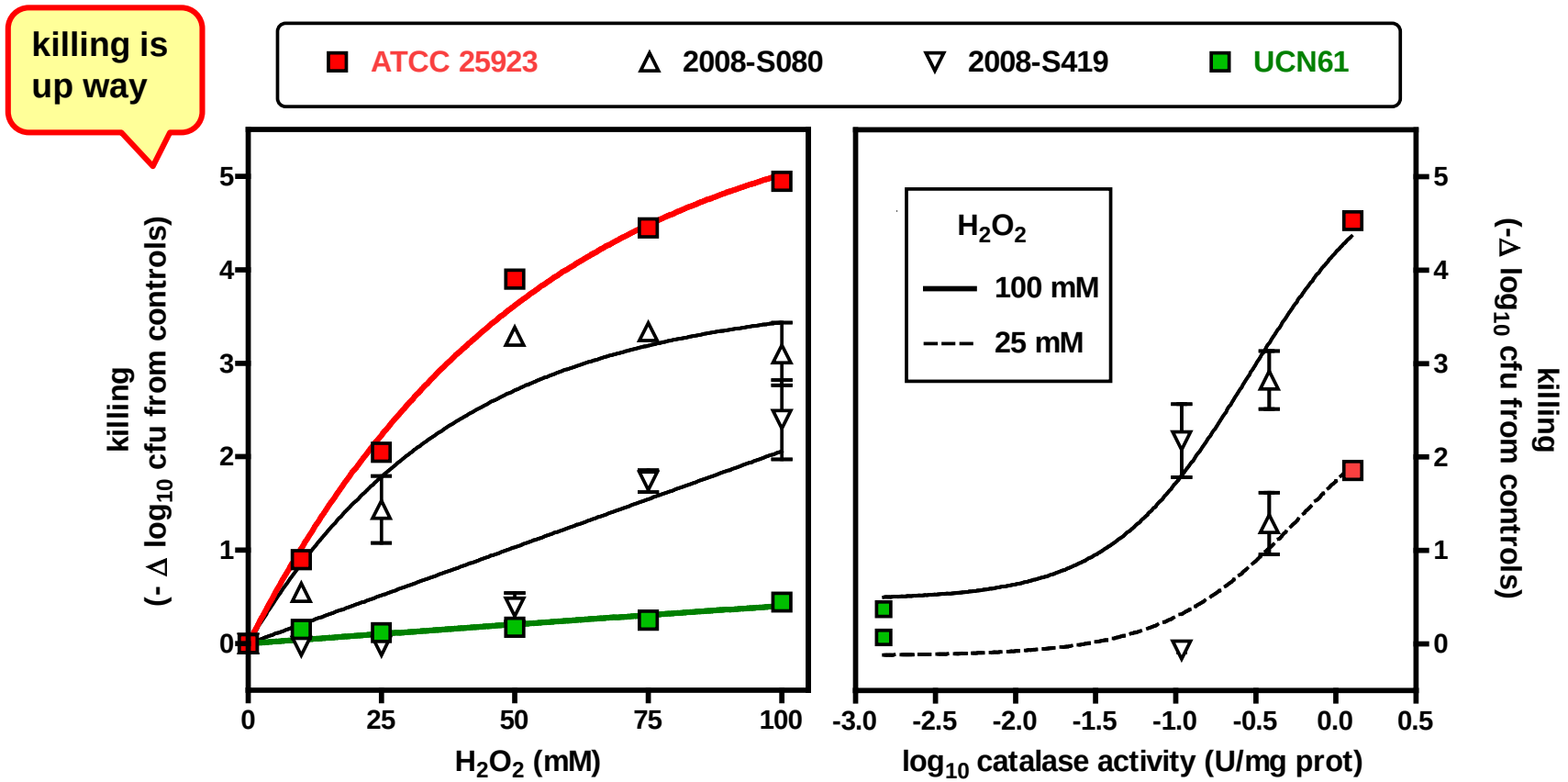
This strain produces almost no catalase

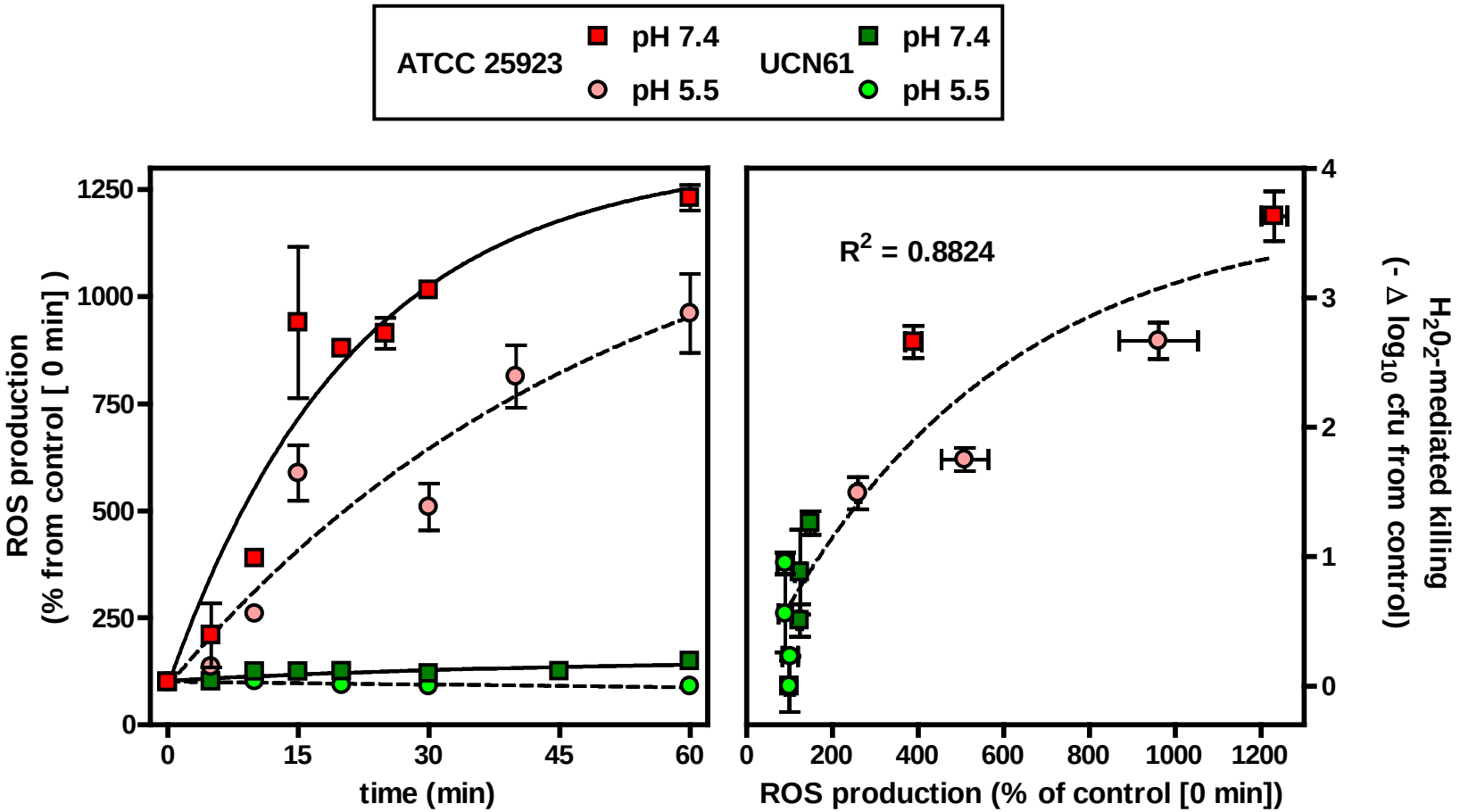
This strain produces a lot of staphyloxanthin

1. Killing of *S. aureus* by exposure to H_20_2



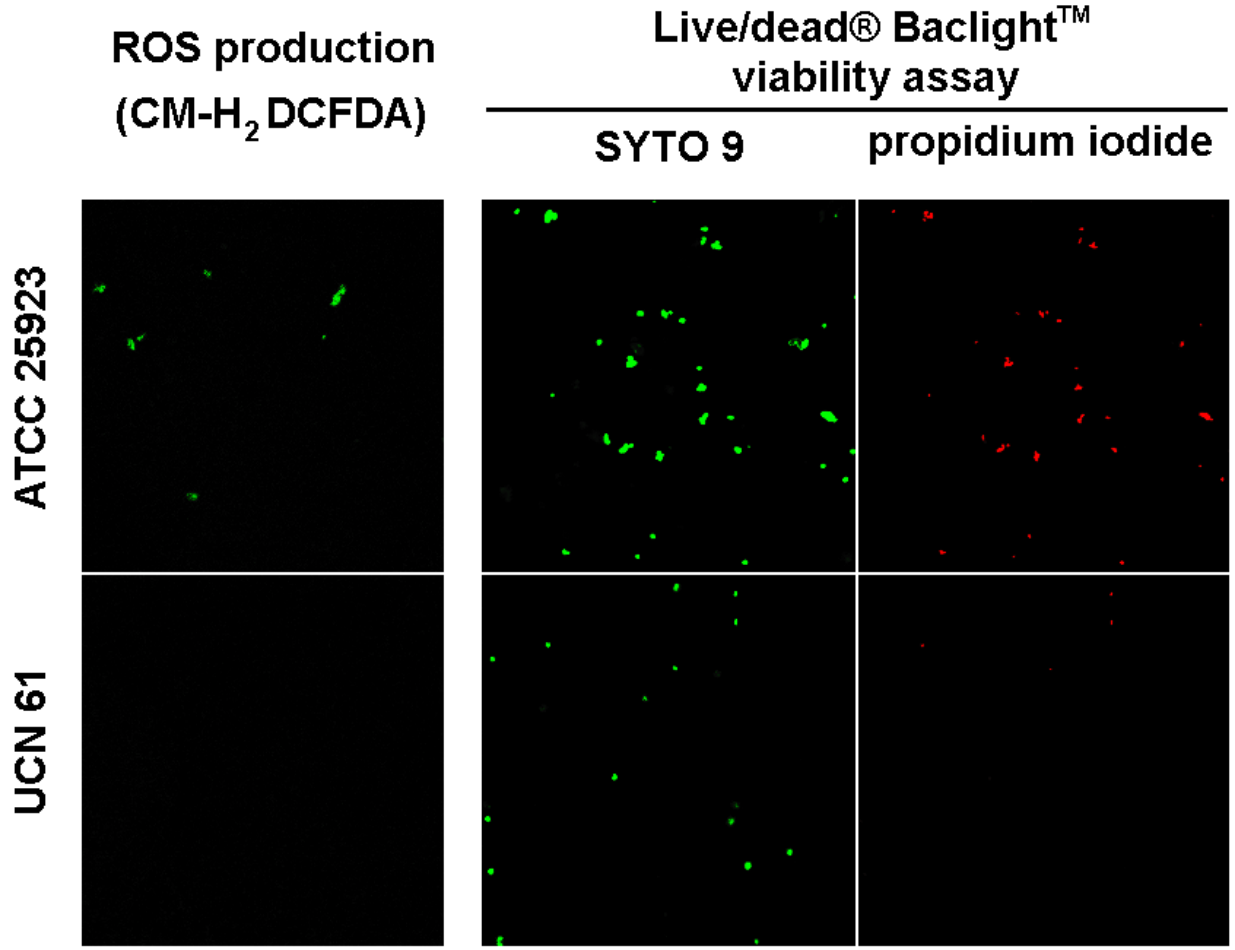
- **Left: Strains with increasing catalase activity (UCN61 < 208-S419 < 2008-S080 < ATCC 25923) exposed to increasing concentrations of H_2O_2 for 45 min, after which surviving bacteria were enumerated by colony counting**
- **Right: same results for two concentrations of H_2O_2 (25 and 100 mM) expressed as a function of the catalase activity of each strain.**

2. Production of ROS by *S. aureus* upon exposure to H_20_2 and correlation with H_20_2-induced killing



- Left: strains loaded with CM-H2DCFDA, exposed to 250 mM H_2O_2 at pH 7.4 or 5.5, and monitored for ROS production
- Right: correlation between bacterial killing and ROS production for the same strains and same pH conditions.

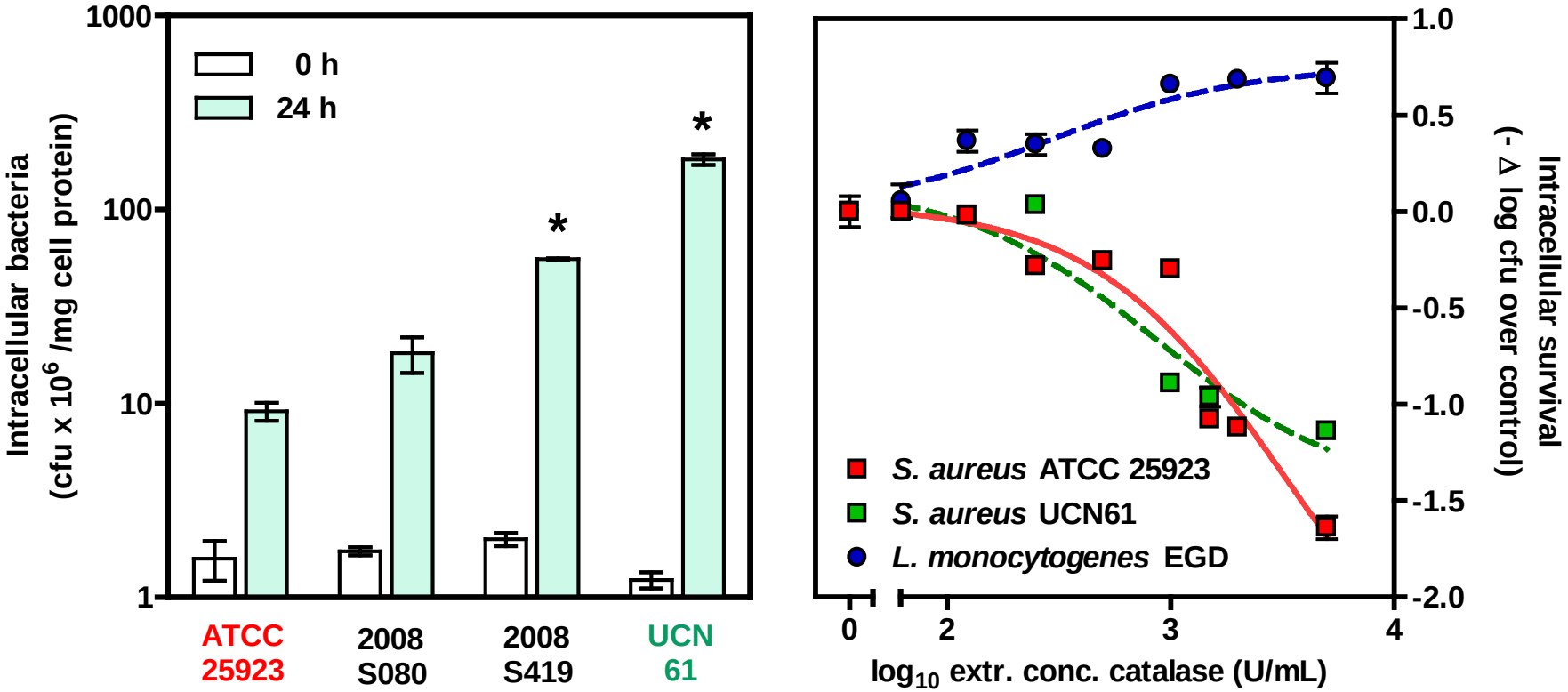
3. Visual evidence of ROS production and bacterial killing of *S. aureus* upon exposure to H_20_2



Confocal microscopy images of bacteria exposed for 30 min at 37°C to 250 mM H_2O_2 at pH 7.4.

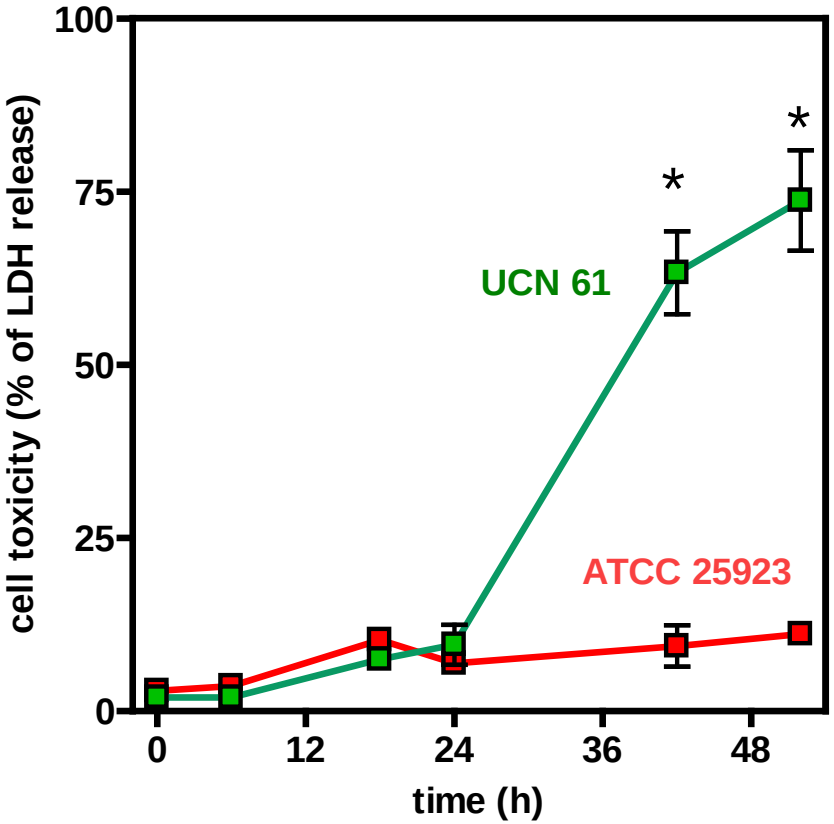
- **Left panels: ROS detection (green signal);**
- **Middle panels: all bacteria (living or dead: green signal);**
- **Right panel: dead bacteria (red signal).**

4. Intracellular growth of *S. aureus* and influence of added catalase (compared to *L. monocytogenes*)



- Left: Intracellular growth of S. aureus strains over 24 post-phagocytosis
- Right: Effect of added catalase on the intracellular survival of *S. aureus* and *L. monocyogenes*

5. Cytotoxicity of phagocytized *S. aureus (*THP-1 monocytes) upon <u>long term</u> intracellular growth



Loss of viability of the monocytes examined by the releas of the cytosolic enzyme lactate dehydogenase (LDH) indicating cell membrane permeabilization.

In a nutshell at this point …

- *S. aureus* strains with a **high catalase activity** are **more readily killed** by exposure to H_2O_2 than those with low activity;
- Strains with **high catalase activity** produce **large amounts ROS**;
- Strains with **high catalase-producing strains** are **less capable of multiplying** in THP 1 monocytes
- Intracellular survival of *S. aureus* is **decreased** by addition of **exogenous catalase.**
- Strain **UCN61** (very **low catalase activity**) readily kills THP-1 monocytes through its **excessive growth** within 24 h whereas ATCC25923 does not.
- Not illustrated: strain **SH1000** (**high** producer of **staphyloxanthin**) is more susceptible to H_2O_2 than strain **UCN61** (low producer of staphyloxanthin) ruling out a major role of this pigment in our findings

What we could not do for a long time…

- We failed, in spite of continuous efforts, to construct *katA* negative mutants and the requisite complemented strains **in the ATCC 25923 background** (but see later)

- We LONG failed to restore catalase activity in the *S. aureus* **UCN61 background** (but see next slides)

  - *Staphylococcus aureus* is known to be a "untransformable bacterium" unless using specific DNA cytosine methyltransferase mutant (DC10B [1]) … or having luck !

  - Catalase may have other critical roles for bacterial survival than its turnover of hydrogen peroxide (maintained or compensated for in the natural strain UCN61)

Note: Isogenic catalase-negative mutants have been described but obtained from the genetically modified SH1000 laboratory strain (restored rsbU activities), which shows an exceptionally large production of staphyloxanthin and is accordingly more resistant than ATCC 25293 to H_2O_2-induced killing [2].

1. Monk IR, Shah IM, Xu M, Tan MW, Foster TJ (2012) Transforming the untransformable: application of direct transformation to manipulate genetically Staphylococcus aureus and Staphylococcus epidermidis. MBio 3. 2012 Mar 20;3(2).
2. Olivier AC, Lemaire S, Van Bambeke F, Tulkens PM, Oldfield E (2009) Role of rsbU and staphyloxanthin in phagocytosis and intracellular growth of Staphylococcus aureus in human macrophages and endothelial cells. J Infect Dis 200: 1367-1370.

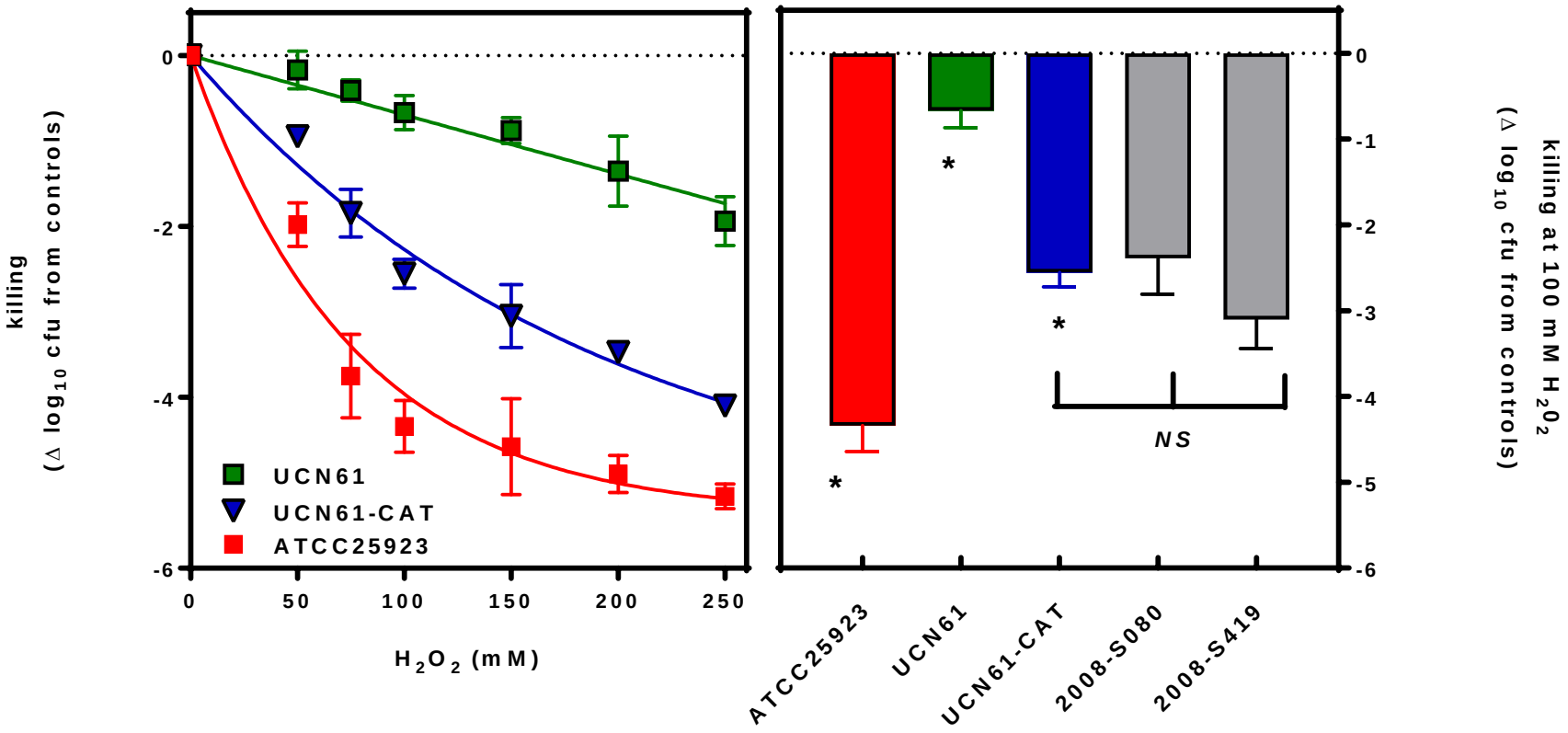
Construction of a UN61 strain with catalase activity

Strain no.	Origin	Catalase activity (U/mg protein)
ATCC 25923	Laboratory reference strains	1.42 ± 0.08
SH1000	Highly pigmented laboratory strain [b]	0.67 ± 0.25 *
2008-S080	Clinical isolate	0.38 ± 0.13 *
2008-S419	Clinical isolate	0.11 ± 0.06 *
UCN61	Naturally occurring mutant of clinical origin	0.0009 ± 0.006 *
UCN61-CAT	**UCN61 complemented with *katA***	**0.77 ± 0.11***

- amplification of katA by PCR and insertion in linearized plasmid pNXR100
- transformation in *S.aureus* RN4220 (a transformable strain deficient in host-restriction barriers) → plasmid pNXR100-KatAcompl
- transformation of UCN61 with pNXR100-KatAcompl by electroporation

* significantly different from strain ATCC 25923 (one way ANOVA with Dunnett multiple comparisons test; P< 0.01)

The *katA*-complemented originally catalase - becomes susceptible to H_2O_2-induced killing



Inclusion of catalase $^{+}$ and catalase $^{-}$ variants in the background of USA300

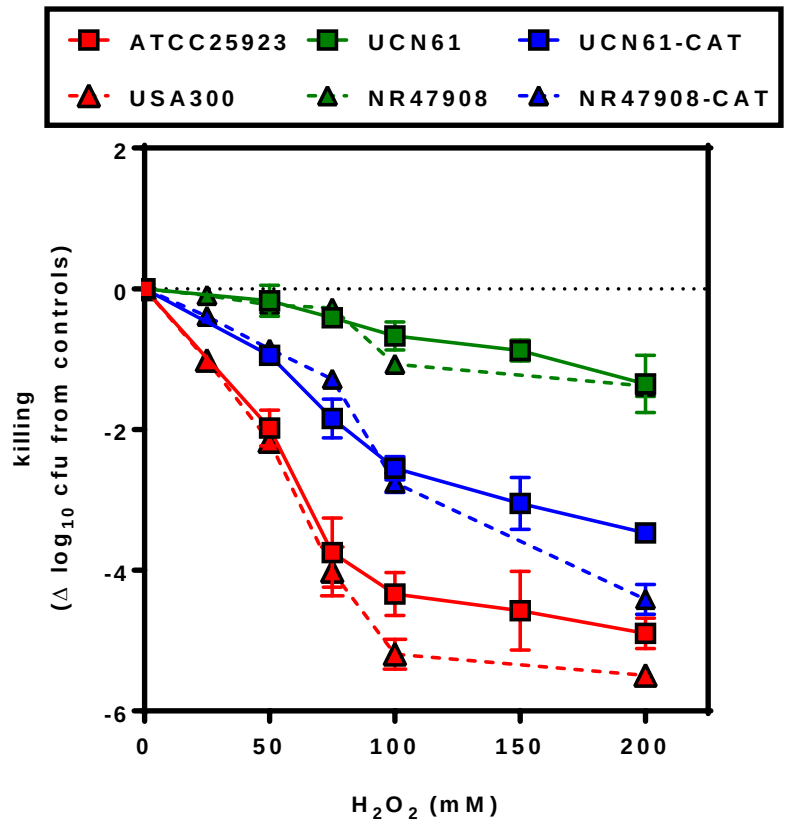
	strain no.	origin	Catalase activity (U/mg protein) [a]
	ATCC 25923	Laboratory reference strains	**1.35 ± 0.10**
	USA300	Clinical isolate	**1.62 ± 0.09**
syngenic	**UCN61**	Naturally occurring *katA* mutant (clinical)	**0.0013 ± 0.06**
	UCN61-CAT	UCN61 complemented with *katA*	**0.89 ± 0.07**
syngenic	**NR47908**	USA-300-derived with *katA* disruption [a]	**0.031 ± 0.016**
	NR47908-CAT	NR47908 complemented with *kat A* [b]	**1.03 ± 0.12**
	SH1000	Highly pigmented laboratory strain	0.67 ± 0.25
	2008-S080	Clinical isolate	0.44 ± 0.11
	2008-S419	Clinical isolate	0.46 ± 0.01

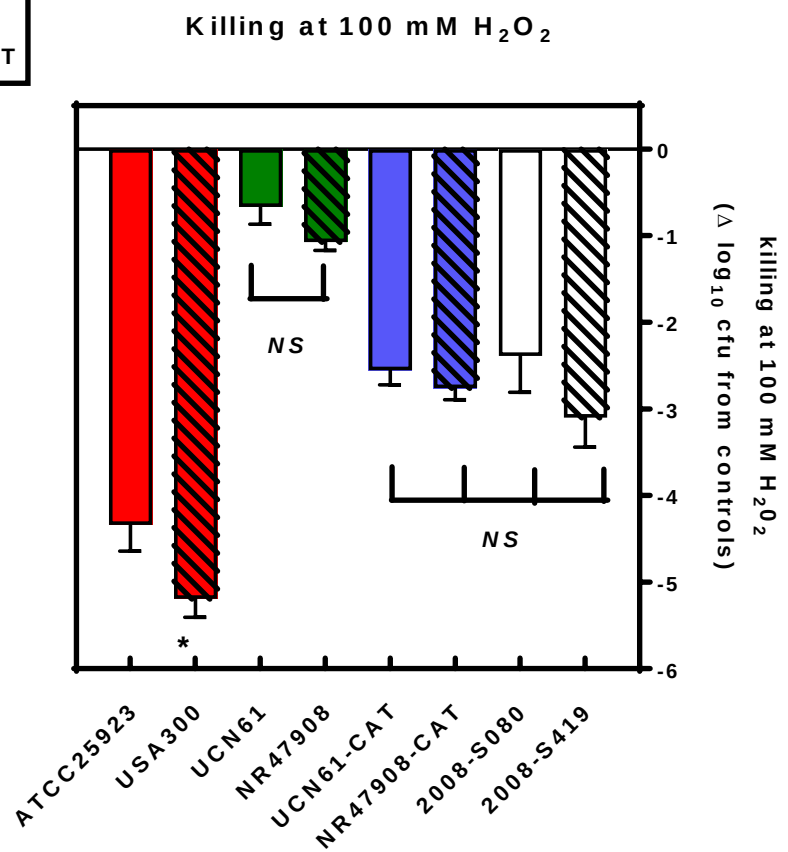
[a] isogenic variant of USA300 obtained from NARSA

[b] constructed by integration of a plasmid containing the *mariner*-based transposon *bursa aurealis* with resistance to erythromycin and integrated in the catalase locus [SAUSA300_1232] of the USA300 strain

Global results for H_2O_2-induced killing …

please, note that we now show killing down way !

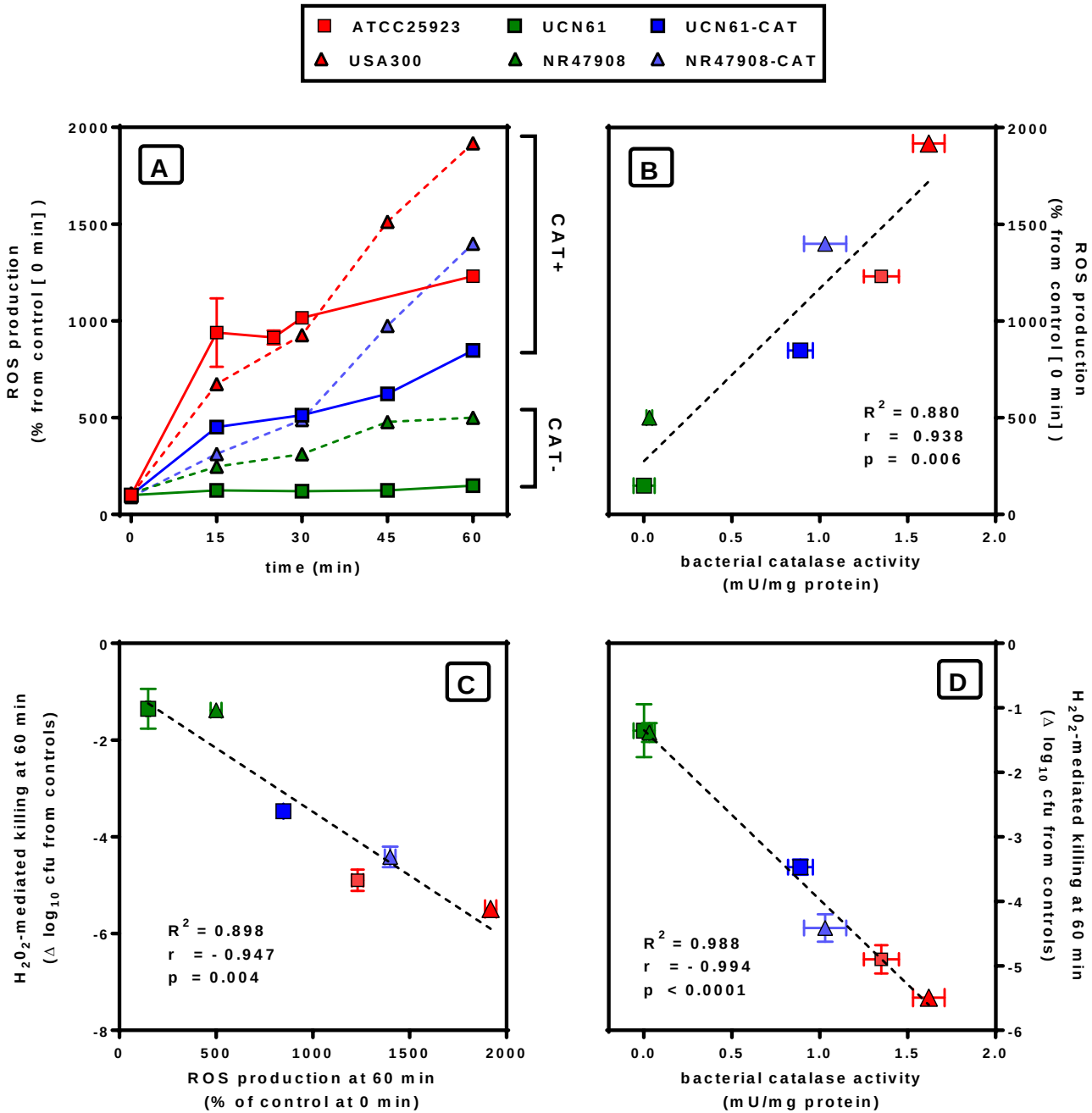




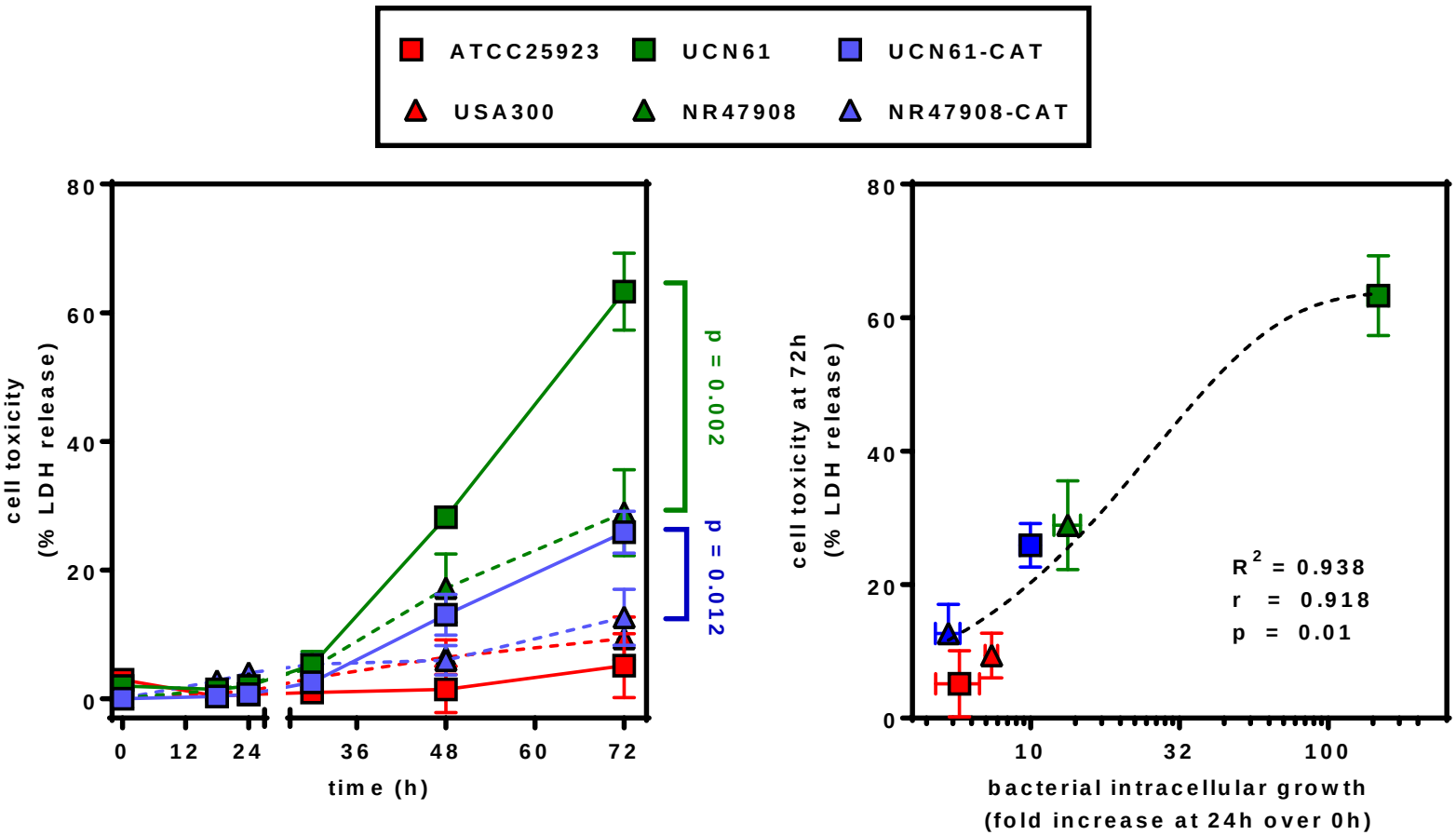
→ the catalase + strains are killed readily
→ the catalase "intermediate" are killed less readily
→ the catalase – strains are killed only very partially

Global results for ROS… and correlations

- ➔ the catalase + strains produce ROS
- ➔ the catalase – strains produce little ROS
- ➔ the catalase "intermediate" strains are intermediate

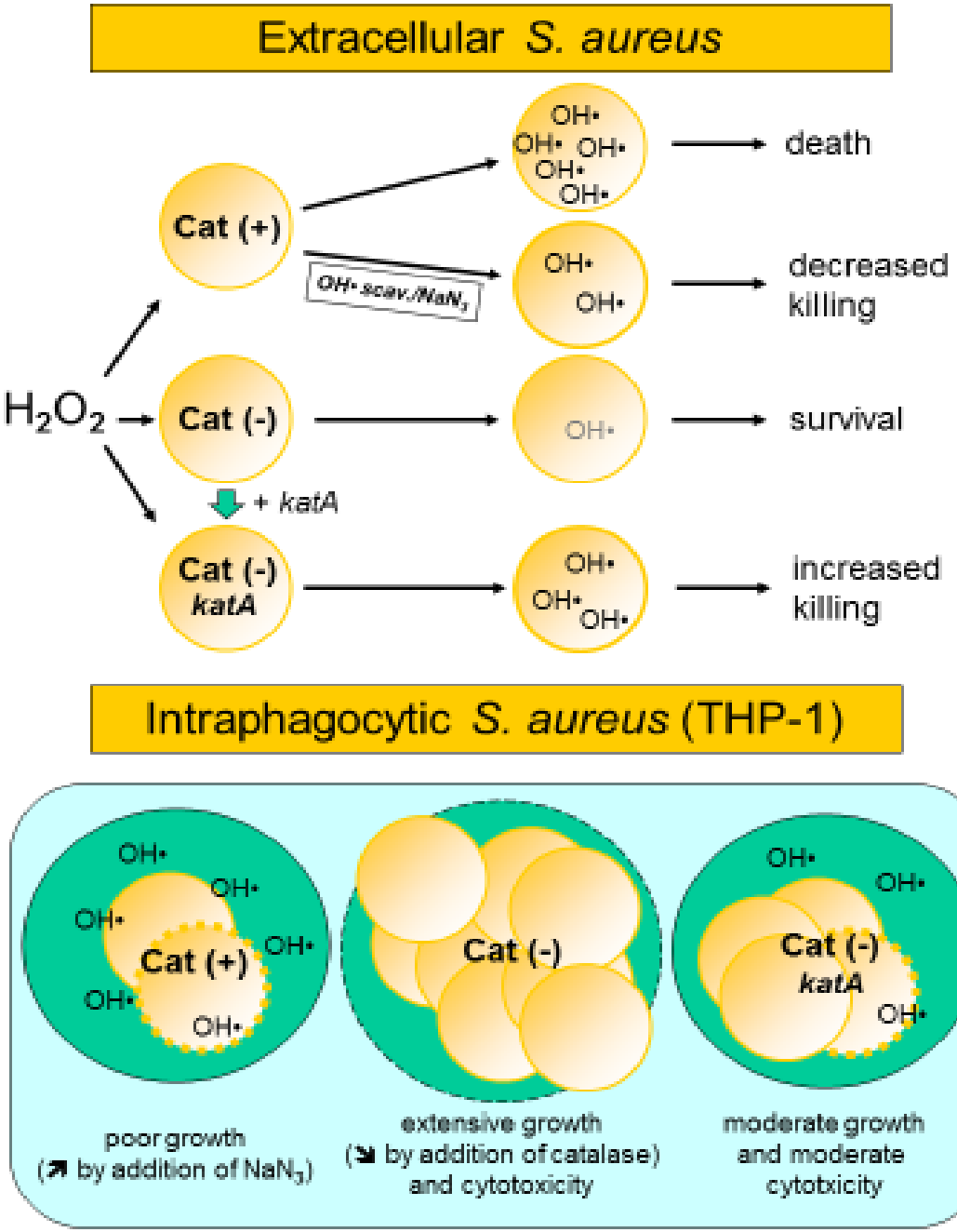


Global results for cytotoxicity and intracellular growth



→ the catalase + strains are not cytotoxic but do not grow
→ the catalase – strains are cytotoxic and grow actively
→ the catalase "intermediate" are intermediate

Graphical summary



Now in words for the essentials…

- A natural catalase-negative clinical isolate was resistant to H_2O_2-induced killing...
- Its *katA*-complemented and catalase-expressing derivative recovered susceptibility to an extent similar to that of clinical catalase-positive strains.
- Conversely, disruption of *katA* (and loss of catalase activity) in the background of USA300 makes the disruptant to resist to H_2O_2-induced killing… but it recovers susceptibility if transfected with a *katA*-containing plasmid

➔ Contrary to current beliefs, catalase may be more detrimental than protective to *Staphylococcus aureus*.

But how could catalase cause bacterial death ?

Our current hypotheses…

1. Catalase activity might involve an **hydroxyl radical** as an intermediate

$$\mathbf{HOOH \rightarrow\ 2\ HO\bullet\ \rightarrow H_2O + \tfrac{1}{2}\ O_2}$$

which could diffuse away from the active site for low fidelity and explain the cidal effects observed…

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which could diffuse away from the active site for low fidelity and explain the cidal effects observed…

2. Catalase is known to also act as an oxidase

$$HOOH + H_2R \rightarrow 2\ H_2O + R$$

and this activity is predominant when the concentration of H_2O_2 is kept at low steady-sate concentrations.[1] Actually, catalase is bactericidal when added to broth containing a system generating H_2O_2 [2].

1. Keilin D, Hartree EF (1945) Properties of catalase. Catalysis of coupled oxidation of alcohols. Biochem J 39: 293-301.
2. Klebanoff SJ (1969) Antimicrobial activity of catalase at acid pH. Proc Soc Exp Biol Med 132: 571-574.

Please, make suggestions !