

# A role for sigma factor $\sigma^E$ in *Corynebacterium pseudotuberculosis* resistance to nitric oxide/peroxide stress

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Pathogenic intracellular bacteria can respond to antimicrobial mechanisms of the host cell through transient activation of stress-responsive genes by alternative sigma ( $\sigma$ ) factors of the RNA polymerase. We evaluated the contribution of the extracytoplasmic function sigma factor  $\sigma^E$  for *Corynebacterium pseudotuberculosis* resistance to stress conditions resembling those found intracellularly during infection. A sigE-null mutant strain ( $\Delta$ sigE) of this bacterium was more susceptible in vitro to acidic pH, cell surface stressors, and biologically relevant concentrations of nitric oxide (NO). The same mutant strain was unable to persist in C57BL/6 mice but remained infective in mice lacking inducible nitric oxide synthase (iNOS), confirming the significance of  $\sigma^E$  for resistance to nitric oxide/peroxide stress in vivo. High-throughput proteomic analysis identified NO-responsive extracellular proteins of *C. pseudotuberculosis* and demonstrated the participation of  $\sigma^E$  in composition of this bacterium's exoproteome.

**Keywords:** *Corynebacterium pseudotuberculosis*, sigma factor, nitric oxide, inducible nitric oxide synthase

## INTRODUCTION

*Corynebacterium pseudotuberculosis*, a pathogenic bacterium belonging to the so-called CMN-group of Actinobacteria, is the etiological agent of various disease manifestations in different hosts, including humans (Dorella et al., 2006; Trost et al., 2010). The high infectious potential of *C. pseudotuberculosis* is dependent on its ability to resist diverse stressful conditions, both as a free-living organism and within the host. Previous reports have suggested that this bacterium can survive in the environment for several months before infecting an animal (Baird and Fontaine, 2007). Once inside the host, *C. pseudotuberculosis* survives phagocytosis to remain as an intracellular parasite within phagocytic cells (Stefańska et al., 2010). This is achieved by overcoming the innate antimicrobial defense mechanisms of the host cells. These cells create a harsh environment inside the phagolysosome by generating reactive oxygen and nitrogen intermediates, via phagocyte oxidase (Phox) and inducible nitric oxide synthase (iNOS), respectively (Nathan and Shiloh, 2000).

Bacteria can respond to different environmental stimuli through switching of the primary sigma ( $\sigma$ ) factor subunit that is associated with core RNA polymerase by alternative  $\sigma$  factors. This confers novel promoter-recognition specificities to the polymerase and provides a mechanism for rapid regulation of different sets of stress-responsive genes (Staron et al., 2009). Proteins of the extracytoplasmic function (ECF) family of alternative  $\sigma$  factors,

such as  $\sigma^E$ , mostly regulate response to cell surface-stresses and have already been shown to control virulence-associated genes in different pathogenic bacteria (Helmann, 2002; Kazmierczak et al., 2005).

Previous studies on *Mycobacterium tuberculosis* transcriptional responses to the stress conditions found within phagocytic cells have suggested the involvement of the ECF sigma factor  $\sigma^E$  in bacterial intracellular survival (Ohno et al., 2003; Schnappinger et al., 2003; Talaat et al., 2004; Fontán et al., 2008). Moreover, sigE has recently been shown to be part of a small set of *M. tuberculosis* genes that are selectively up-regulated in response to nitrosative stress (Voskuil et al., 2011).

In this study with *C. pseudotuberculosis*, we show that  $\sigma^E$  is indeed required for resistance to in vitro-generated, biologically relevant concentrations of nitric oxide (NO). Moreover, we also demonstrate that this regulatory protein plays a role in resistance to combined NO/peroxide stress faced by bacteria during infection.

## MATERIALS AND METHODS

### BACTERIAL STRAINS AND GROWTH CONDITIONS

*Corynebacterium pseudotuberculosis* strains were routinely maintained in Brain Heart Infusion broth or in BHI 1.5% bacteriological agar plates, at 37°C. Tween-80 was added to the broth cultures at 0.05% to prevent cell clumping. When necessary,

the antibiotic kanamycin was used at 25 µg/ml. *Escherichia coli* strains were maintained in Luria–Bertani medium and employed in DNA manipulation experiments, according to standard protocols. Ampicillin (100 µg/ml) and kanamycin (50 µg/ml) were used where appropriate.

A chemically defined medium (CDM), previously optimized for growth of *C. pseudotuberculosis* (Moura-Costa et al., 2002), was used in all the experiments that included stress-generating agents. The composition of the CDM was as follows: auto-claved phosphate buffer pH = 7.4 [ $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$  (12.93 g/l),  $\text{KH}_2\text{PO}_4$  (2.55 g/l),  $\text{NH}_4\text{Cl}$  (1 g/l),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.20 g/l),  $\text{CaCl}_2$  (0.02 g/l), and 0.05% (v/v) Tween-80]; 4% (v/v) MEM Vitamins Solution 100× (Invitrogen); 1% (v/v) MEM Amino Acids Solution 50× (Invitrogen); 1% (v/v) MEM Non-Essential Amino Acids Solution 100× (Invitrogen); and 1.2% (w/v) filter-sterilized glucose.

#### CONSTRUCTION OF A sigE-NULL MUTANT STRAIN OF C. PSEUDOTUBERCULOSIS

A conserved region of the sigE gene of *C. pseudotuberculosis* (GenBank: ADL20681.1) was initially isolated by a strategy employing degenerate primers. Basically, the amino acid sequences of the  $\sigma^E$  factors of the bacteria *M. tuberculosis* (Rv1221), *C. diphtheria* (DIP0994), *C. glutamicum* (NCgl1075), and *C. efficiens* (CE1177) were retrieved from public databases and aligned using the ClustalW tool. Highly conserved regions were identified in the  $\sigma^E$  factors of these bacteria and degenerate primers were designed according to the coding sequences of these factors, such that it would be possible to amplify a partial region of the sigE gene in all genomes analyzed. The primer pair sigE#1: GGMACCGCAGCDTTCGACGC and sigE#2: CGTCCRCGGTGRATWCGGGA was used for amplification of an expected 490 bp internal fragment of the sigE gene of *C. pseudotuberculosis*. PCR products around the expected size were purified from agarose gels, ligated into the vector pCR2.1 TOPO (Invitrogen), and sequenced according to standard protocols.

A plasmid carrying a fragment of the sigE gene was introduced into the wild-type strain 1002 of *C. pseudotuberculosis* by electroporation. This plasmid functions as a suicide vector for this bacterium, as it does not carry a functional corynebacterial replication origin. *C. pseudotuberculosis* clones that underwent recombination were selected by kanamycin resistance (25 µg/ml). Confirmation of recombination events in the *C. pseudotuberculosis* genome was obtained by Southern blot analysis using a [ $\alpha^{32}\text{-P}$ ]dCTP radiolabeled probe for the sigE gene, or through PCR reactions employing different combinations of primers that align in the start and stop codons of sigE with primers that align within the inserted plasmid: sigE#3-ATGACATCGAACAGTGGTTC/sigE#4-TTAGTGGGACATCGGTAGG; Kan#1-ATGATTGAACAAGATGG ATTG/Kan#2-TTAATAATTCAGAAGAACTC; M13F/M13R (Invitrogen; data not shown).

#### IN VITRO RESISTANCE TO STRESS-GENERATING AGENTS

*Corynebacterium pseudotuberculosis* strains were grown in CDM to early exponential phase ( $\text{OD}_{540\text{nm}} = \sim 0.1$ ), cultures were split into several aliquots and then incubated separately with

different concentrations of various stress-generating agents, as follows: osmotic stress (NaCl, 0.5 and 1 M); acidic stress (HCl, to pH 4.5 and 5.5); detergent stress (SDS, 0.01% w/v and 0.05%); lysozyme, 500 and 750 µg/ml; alcoholic stress (ethanol, 2.5 and 5%); oxidative stress ( $\text{H}_2\text{O}_2$ , 1, 10, and 50 mM); nitric oxide stress (DETA/NO – diethylenetriamine NON-Oate, 0.1 and 1 mM). For starvation stress, CDM was prepared with low concentrations of glucose: 0.3 and 0.15%. For heat and cold shocks, cultures were incubated at 50 or 4°C for 30 min, and then returned to normal growth at 37°C.

Growth of the control and stressed cultures of *C. pseudotuberculosis*, of both wild-type (1002) and mutant ( $\Delta\text{sigE}$ ) strains, was monitored for 24 h in a LabSystems iEMS Absorbance Plate Reader (Thermo Fisher), at  $\text{OD}_{540\text{nm}}$ . Results were plotted using the GraphPad Prism software (GraphPad Software, Inc); the integrals of the areas under the curves (AUC) were determined, and a growth index (GI) was then calculated, as follows:  $\text{GI} (\%) = [(\text{AUC}_{\text{Treated culture}}/\text{AUC}_{\text{Control culture}}) \times 100]$ . Experiments were performed at least in triplicate.

#### NITRIC OXIDE DETECTION

Steady-state concentrations of NO generated by the NO-donor DETA/NO in the cell culture medium were determined amperometrically using an NO-specific electrode (ISO-NOP, World Precision Instruments, Inc.) attached to an NO meter (ISO-NO Mark II, WPI). Briefly, the electrode was immersed in CDM and the electrode current allowed to stabilize under conditions identical to those used in the cell culture experiments before addition of different concentrations of DETA/NO and recording of the electrode response. Stock solutions of DETA/NO (Sigma-Aldrich) were prepared fresh and kept on ice in the dark, as described (Feelisch, 1998). Currents in pA were converted into NO concentrations by comparison to a standard calibration curve generated from either copper(I)-mediated decomposition of S-nitroso-N-acetyl-d,l-penicillamine or from iodide-mediated nitrite reduction, under otherwise identical conditions (Davies and Zhang, 2008).

#### INFECTION OF MOUSE MACROPHAGES WITH C. PSEUDOTUBERCULOSIS

Bone-marrow derived macrophages (BMMs) were obtained from the femurs and tibias of C57BL/6 or iNOS knockout mice (iNOS<sup>-/-</sup>), according to a standardized protocol (Carvalho et al., 2011). Gamma ( $\gamma$ )-interferon was added at 20 U/well for macrophage activation. A Neutral Red assay was used to evaluate macrophage viability following *C. pseudotuberculosis* infection (MOI 5:1), as described (McKean et al., 2007).

#### CORYNEBACTERIUM PSEUDOTUBERCULOSIS GROWTH AND PERSISTENCE IN C57BL/6 AND iNOS<sup>-/-</sup> MICE

C57BL/6 or iNOS<sup>-/-</sup> mice were infected intraperitoneally with  $10^6$  colony forming units (CFU) of the wild-type (1002) or mutant ( $\Delta\text{sigE}$ ) strain of *C. pseudotuberculosis*. On days 1 and 3 post-infection, animals were sacrificed and bacterial loads in the spleens were enumerated.

## EXTRACTION OF *C. PSEUDOTUBERCULOSIS* EXTRACELLULAR PROTEINS FOLLOWING NO-TREATMENT AND COMPARATIVE EXOPROTEOME PROFILING

The 1002 (wt) and  $\Delta$ sigE strains of *C. pseudotuberculosis* were grown in CDM to mid-exponential phase, cultures were split into two aliquots and 100  $\mu$ M of the NO-donor DETA/NO were added to one aliquot of each strain. NO-treated cultures along with control cultures were further incubated for 1 h at 37°C. At this point, extracellular proteins were extracted by the three-phase partitioning technique and identified/quantified by a high-throughput proteomic strategy, based on a recently introduced method of liquid chromatography–mass spectrometry acquisition (LC–MS<sup>E</sup>), exactly as previously described (Pacheco et al., 2011). Biological function annotations for the identified proteins were retrieved from the gene ontology (GO) database, using the web tool AmiGO<sup>1</sup> (Carbon et al., 2009). Protein regulation data were obtained from the CoryneRegNet v6.0 Database<sup>2</sup> (Pauling et al., 2011).

## RESULTS

### A sigE-NULL MUTANT STRAIN OF *C. PSEUDOTUBERCULOSIS* IS MORE SENSITIVE TO CELL SURFACE STRESSORS, ACIDIC PH, AND IN VITRO-GENERATED NITRIC OXIDE

In order to evaluate the role played by the ECF sigma factor  $\sigma^E$  in *C. pseudotuberculosis* resistance to stress conditions faced during intracellular infection, a sigE-null mutant strain of this bacterium ( $\Delta$ sigE) was generated by homologous recombination in the parental strain 1002 (wt). Both strains were submitted to a series of stress-generating agents in vitro, which aimed at mimicking conditions found by bacterial pathogens in the intraphagosomal environment (Rohde et al., 2007; Ehrh and Schnappinger, 2009; Schaible, 2009).

Mutation of sigE in *C. pseudotuberculosis* did not alter cell growth and morphology under normal conditions (Figure 1).

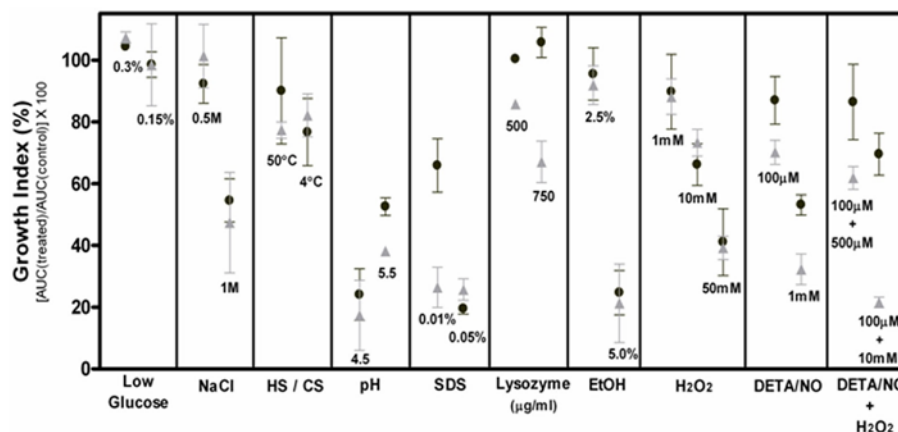
Growth of the  $\Delta$ sigE strain was also comparable to the 1002 (wt) strain under conditions of nutrient starvation (limiting glucose), and under osmotic, thermal, alcoholic, and oxidative stresses (Figure 1). On the other hand, the mutant strain was more sensitive to an acidic pH that resembles that found within an activated macrophage (pH = 5.5), and to cell surface stressors, namely SDS and lysozyme treatments (Figure 1), corroborating previous studies on the role of  $\sigma^E$  in resistance to environmental-stresses in other corynebacteria and mycobacteria (Manganelli et al., 2001; Park et al., 2008). Interestingly, growth of the  $\Delta$ sigE strain was also more affected than that of the wt strain following exposure to different concentrations of the NO-donor DETA/NO (Figures 1 and 2A). NO electrode measurements indicated that the concentrations of the NO-donor used in our experiments, 0.1 and 1 mM, resulted in steady-state NO concentrations of ~500 nM and 5  $\mu$ M, respectively. The effect of these concentrations on growth of the two *C. pseudotuberculosis* strains was apparently more due to a bacteriostatic rather than bactericidal action of NO (Figure 2C), as reported for other bacterial pathogens (Ogawa et al., 2001; Voskuil et al., 2011). Notably, the  $\Delta$ sigE strain was much more sensitive to the combination of the NO-donor and H<sub>2</sub>O<sub>2</sub> (Figures 1 and 2B), reinforcing the requirement of  $\sigma^E$  for *C. pseudotuberculosis* resistance to combined NO/peroxide stress.

### $\sigma^E$ IS REQUIRED FOR *C. PSEUDOTUBERCULOSIS* RESISTANCE TO NO-STRESS DURING INFECTION

The participation of  $\sigma^E$  in *C. pseudotuberculosis* resistance to the conditions found within the host was first evaluated by infection of C57BL/6 mice with the wt and mutant strains of this bacterium, followed by analysis of bacterial persistence in mouse spleens. After 3 days of infection, the  $\Delta$ sigE strain of *C. pseudotuberculosis* was virtually undetectable in the spleens of C57BL/6 mice, whereas the 1002 (wt) strain still persisted (Figure 3A). We then evaluated whether nitrosative stress was a determining condition for the lowered persistence of the  $\Delta$ sigE mutant in the host. For this, we infected C57BL/6 or

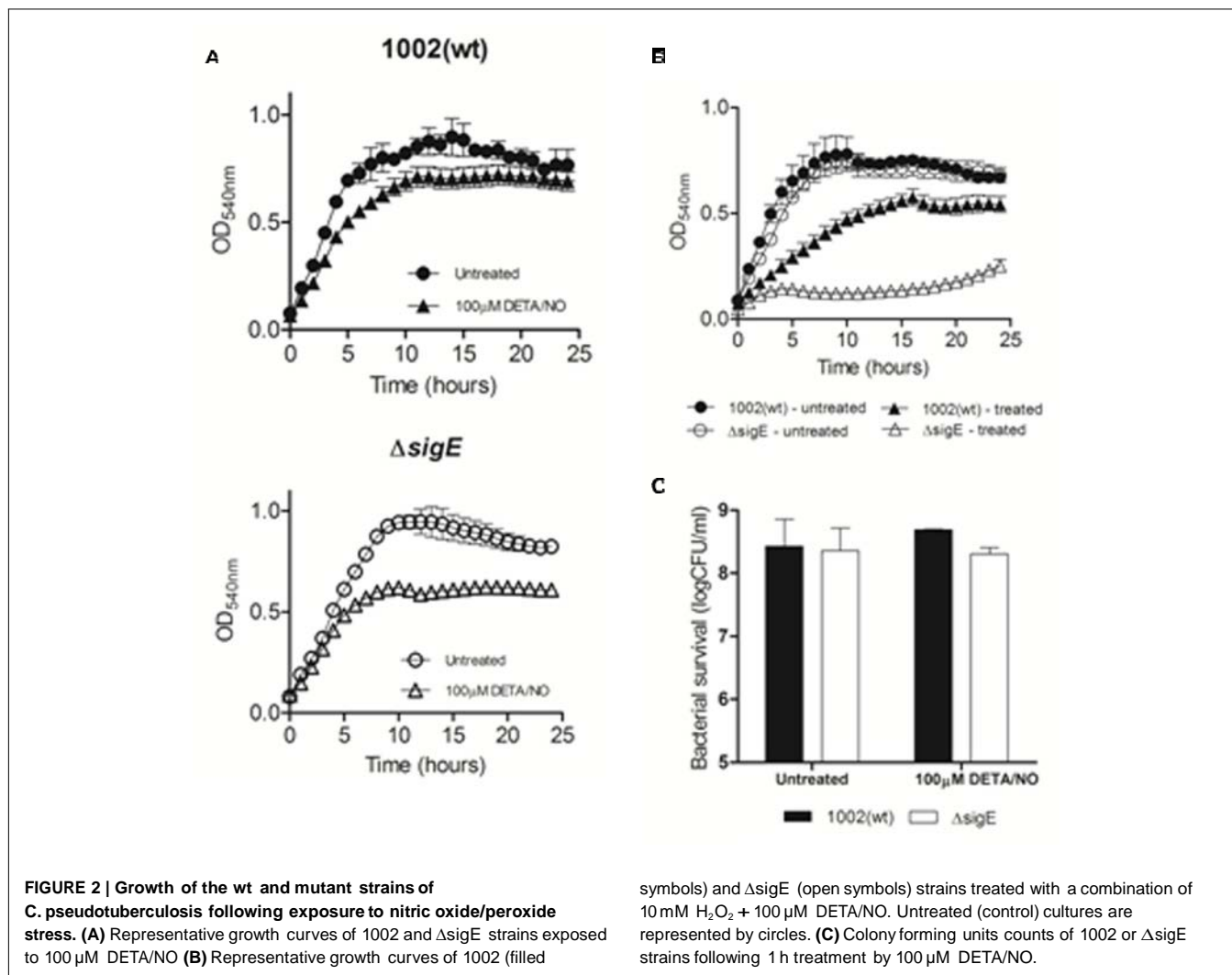
<sup>1</sup><http://amigo.geneontology.org>

<sup>2</sup><http://www.coryneregnet.de/>



**FIGURE 1 | Differential susceptibilities of the parental (1002) and mutant ( $\Delta$ sigE) strains of *C. pseudotuberculosis* to different stress conditions in vitro. Average percent growth  $\pm$  SD of 1002 (black circles) and  $\Delta$ sigE (gray**

triangles) strains exposed to various stress conditions in vitro (see text for details), in comparison to non-treated controls. HS, heat shock; CS, cold shock.



iNOS knockout (iNOS<sup>-/-</sup>) mice, which are unable to produce NO in the intraphagosomal environment, and compared bacterial loads in mouse spleens 3 days post-infection. Again, the *C. pseudotuberculosis* strain lacking  $\sigma^E$  was unable to persist in C57BL/6 wild-type mice (**Figure 3B**); nevertheless, the  $\Delta$ sigE mutant displayed a surprisingly high ability to survive in the iNOS<sup>-/-</sup> animals, following an initial 10<sup>6</sup> CFU inoculum (**Figure 3B**).

Wild-type *C. pseudotuberculosis* has been shown to possess a profound capability to kill macrophages in culture (Stefańska et al., 2010), but this natural ability is also affected in the  $\Delta$ sigE strain (**Figure 3C**). However, only ca. 30% of iNOS<sup>-/-</sup> macrophages survived after 4 h infection with this mutant (**Figure 3C**). This corroborated the importance of  $\sigma^E$  in *C. pseudotuberculosis* resistance to NO generated intracellularly.

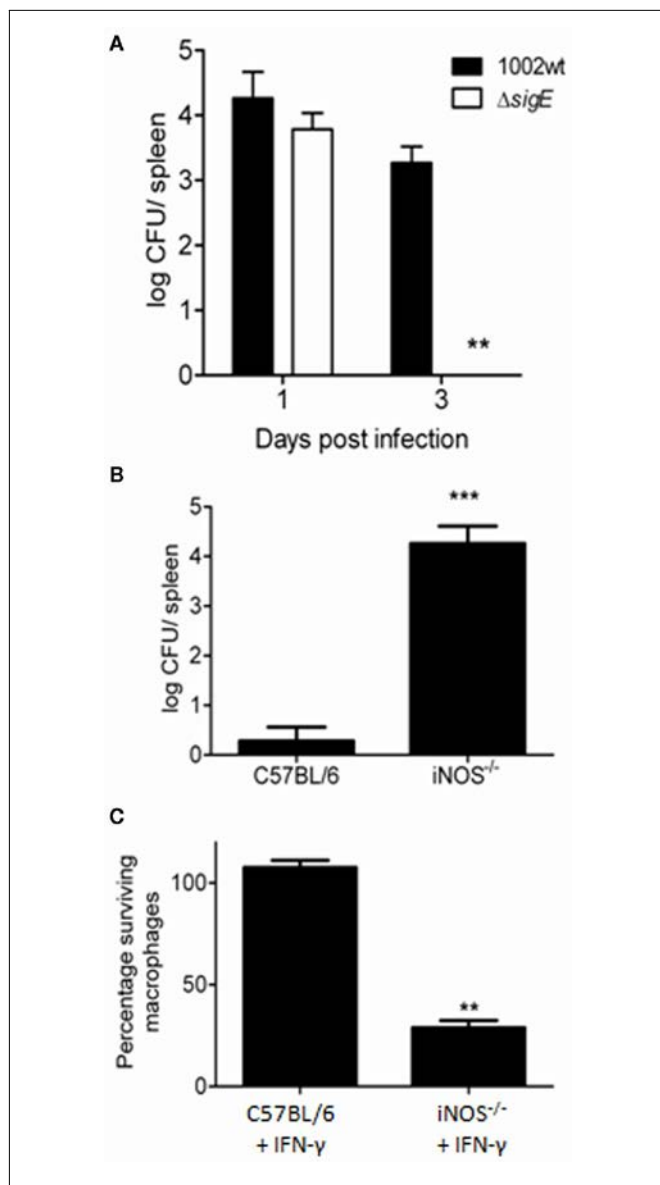
#### ALTERATIONS OF THE *C. PSEUDOTUBERCULOSIS* EXOPROTEOME IN RESPONSE TO NO-STRESS

The extracellular proteomes of the 1002 (wt) and  $\Delta$ sigE strains of *C. pseudotuberculosis* were profiled by a high-throughput

proteomics method (LC-MS<sup>E</sup>), before and after treatment by 100  $\mu$ M of the NO-donor DETA/NO.

In total, 281 extracellular proteins were confidentially identified by this strategy, in the four groups studied: (i) 1002 (wt) untreated; (ii) 1002 (wt) + DETA/NO; (iii)  $\Delta$ sigE untreated; (iv)  $\Delta$ sigE + DETA/NO. This represented 104 different extracellular proteins of *C. pseudotuberculosis* (**Figure 4A**). Proteins exported after NO-treatment could be detected in both 1002 (wt) and  $\Delta$ sigE strains (**Figure 4A**). The mutant strain exported a higher number of proteins in comparison to the wt strain, under normal growth conditions, and after NO-stress (**Figure 4A**). The majority (67%) of these differential proteins of the  $\Delta$ sigE strain are predicted in silico to be truly exported proteins (data not shown). Moreover, the proteins commonly identified in the four groups presented very similar concentrations in all samples (**Figure 4B**) showing a high reproducibility of the methods used for extraction and identification of the exoproteins. This demonstrates that the qualitative differences observed between the exoproteomes are in fact due to biological variations of the extracellular proteomes.

Three proteins were commonly identified in the 1002 (wt) and  $\Delta$ sigE strains exclusively after treatment by NO (**Figure 4A**). Only



**FIGURE 3 | Differential persistence of the  $\Delta sigE$  mutant of *C. pseudotuberculosis* during infection of wild-type and iNOS<sup>-/-</sup> mice. (A)** The  $\Delta sigE$  strain is rapidly eliminated, becoming almost undetectable in the spleens of C57BL/6 mice 3 days after inoculation with  $10^6$  CFU. **(B)** Bacterial loads in spleens of C57BL/6 or iNOS<sup>-/-</sup> mice, 3 days post-infection with the  $\Delta sigE$  strain of *C. pseudotuberculosis*. **(C)** Viability of macrophages obtained from either C57BL/6 or iNOS<sup>-/-</sup> mice, 4 h post-infection with the  $\Delta sigE$  strain of *C. pseudotuberculosis*. \*\*t-Test  $p < 0.001$ ; \*\*\*t-test  $p < 0.0001$ .

a few proteins were differentially exported in the 1002 (wt) strain in response to NO, compared to the mutant strain (Figure 4A). These included proteins predicted to be involved in metal ion transport and cell redox homeostasis. A putative dioxygenase was also specifically detected in the exoproteome of the wt strain (Table 1). On the other hand, 17 differential proteins could be detected in the extracellular proteome of the  $\Delta sigE$ -null strain following NO-stress (Figure 4A). Metal ion transport and cell redox homeostasis

were again highly represented predicted biological functions in this protein set (Table 1). Some of these proteins have already been seen to participate in *C. pseudotuberculosis* responses to other environmental stress conditions, according to the CoryneRegNet Database (Table 1).

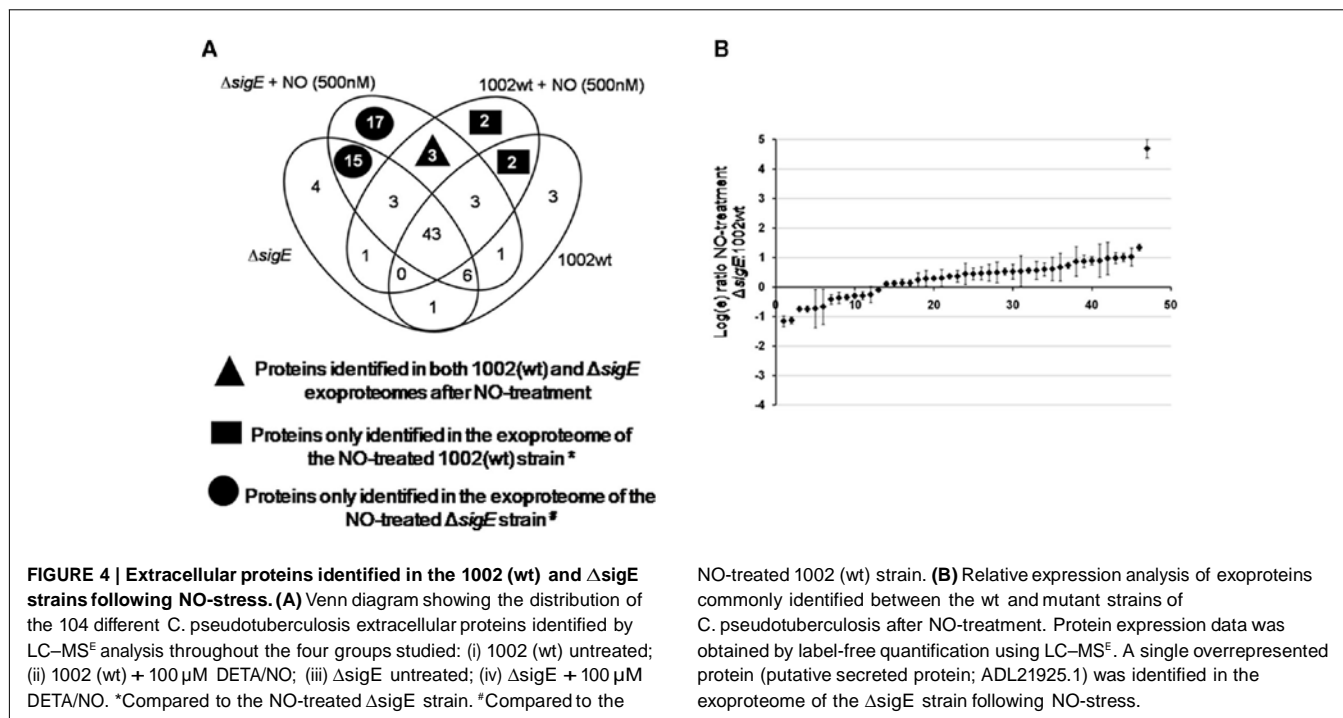
A few proteins primarily considered to have a cytoplasmic location have been identified following NO-stress (Table 1). These included the chaperonin GroEL, which was found in the exoproteomes of both wt and  $\Delta sigE$  strains of *C. pseudotuberculosis*, and the proteins glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and dnaK, differentially exported only in the mutant strain (Table 1). These cytoplasmic proteins have been consistently identified in recent exoproteome studies of various bacteria (Sengupta et al., 2010; Dreisbach et al., 2011). Besides, environmental cues such as osmotic stress and host contact have been shown to induce exportation of some of these proteins (Chitlaru et al., 2007; Mattinen et al., 2007; Pumirat et al., 2009). However, the molecular mechanisms that account for non-classical (leaderless) secretion of specific cytoplasmic proteins is still to be completely understood (Pasztor et al., 2010; Yang et al., 2011). Moonlighting roles have already been demonstrated for these proteins while in the extracellular environment, including adhesion to host cells and evasion of host's immune mechanisms (Matta et al., 2010; Jin et al., 2011).

Noticeably, while most of the proteins commonly identified between the exoproteomes of the NO-treated strains presented very similar concentrations (Figure 4B), a single protein annotated as a "putative secreted protein" (ADL21925.1) was highly secreted only in the  $\Delta sigE$  strain. This protein has also been shown to be differentially regulated during osmotic stress, according to CoryneRegNet.

## DISCUSSION

Pathogenic bacteria that reside within phagocytic cells face nitrosative stress due to the action of iNOS, which delivers NO to the intraphagosomal compartment (Nathan and Shiloh, 2000; Ehrt and Schnappinger, 2009). NO is a highly reactive radical which in high concentrations can lead to metabolic arrest and damage of cellular components, including membranes and DNA, thus inhibiting bacterial growth (Ogawa et al., 2001). Through combination with reactive oxygen species, considerably more reactive antimicrobial agents can be generated, such as peroxynitrite (ONOO<sup>-</sup>; Ehrt and Schnappinger, 2009). To survive such stressful conditions found in the intracellular environment, bacteria need to be able to rapidly respond to environmental cues through global changes in gene expression; this role is played by alternative sigma factors of the bacterial RNA polymerase (Helmann, 2002).

Previous studies with *M. tuberculosis* have consistently shown that the gene coding for the extracytoplasmic function sigma factor  $\sigma^E$  is up-regulated during intracellular infection (Ohno et al., 2003; Schnappinger et al., 2003; Talaat et al., 2004; Fontán et al., 2008). More recently, the sigE gene was shown to participate in a specific transcriptional response of *M. tuberculosis* following NO-treatment in vitro (Voskuil et al., 2011). Taken together, these studies strongly suggest that  $\sigma^E$  may be necessary for *M. tuberculosis* to cope with reactive nitrogen species



**Table 1 | Proteins identified in the exoproteomes of the 1002 (wt) and  $\Delta sigE$  strains of *C. pseudotuberculosis* following NO-stress<sup>a</sup>.**

Protein ID	Protein description	GO biological process annotation
<b>PROTEINS IDENTIFIED IN BOTH 1002 (wt) AND <math>\Delta sigE</math> EXOPROTEOMES AFTER NO-TREATMENT</b>		
ADL20157.1	Conserved hypothetical protein Cp1002_0254	Growth of symbiont in host cell
ADL21673.1	Chaperonin GroEL	Response to stress
<b>PROTEINS ONLY IDENTIFIED IN THE EXOPROTEOME OF THE NO-TREATED 1002 (wt) STRAIN<sup>b</sup></b>		
ADL20218.1	ABC-type metal ion transport system	Metal ion transport; cell adhesion; pathogenesis
ADL20030.1	Glyoxalase/dioxygenase	Metabolic process
ADL21047.1	Thiol peroxidase	Response to nitrosative stress; cell redox homeostasis; pathogenesis
<b>PROTEINS ONLY IDENTIFIED IN THE EXOPROTEOME OF THE NO-TREATED <math>\Delta sigE</math> STRAIN<sup>c</sup></b>		
ADL20192.1	ABC-type transporter <sup>d</sup>	Iron ion transport
ADL20337.1	ABC transporter substrate-binding protein <sup>d</sup>	Metal ion transport
ADL21101.1	Alkyl hydroperoxide reductase subunit C	Cell redox homeostasis; oxidation–reduction process; response to nitrosative stress
ADL21757.1	Chaperone protein DnaK <sup>d</sup>	Cellular response to superoxide; growth; response to heat
ADL21709.1	Nitrite reductase periplasmic cytochrome c552	Nitrogen compound metabolic process
ADL20019.1	Periplasmic zinc-binding protein troA	Cation transport
ADL21903.1	ABC-2 type transporter family protein <sup>d</sup>	Transport
AEK49214.1	ABC transporter glutamine-binding protein glnH	Growth
ADL19943.1	Penicillin-binding protein A	Cell wall assembly
ADL21114.1	Phosphocarrier protein HPr	Phosphoenolpyruvate-dependent sugar phosphotransferase system
ADL21286.1	Serine/threonine protein kinase	Protein autophosphorylation
ADL21721.1	Fructose-bisphosphate aldolase	Glycolysis
ADL20991.1	GAPDH	Growth

GO, gene ontology.

<sup>a</sup>Only the differential proteins for which it was possible to assign a Biological Process annotation are included in this table.

<sup>b</sup>In comparison to the  $\Delta sigE$  mutant strain.

<sup>c</sup>In comparison to the 1002 (wt) strain.

<sup>d</sup>These proteins have already been shown to be differentially regulated in *C. pseudotuberculosis* in response to either heat shock, osmotic, or acidic stresses, according to the CoryneRegNet Database v6.0 (<http://www.coryneregnet.de/>).

within the host cell, but this possibility has not been specifically investigated yet.

In this study, we show that  $\sigma^E$  is indeed required for full resistance to nitrosative stress in the intracellular actinobacterium *C. pseudotuberculosis*. Biologically relevant concentrations of NO, generated here by the NO-donor DETA/NO, importantly affected growth of this bacterium, similarly to what has been reported for *M. tuberculosis* (Voskuil et al., 2003, 2011). Growth impairment was much more pronounced in a sigE-null mutant strain of *C. pseudotuberculosis*, following exposure to both low and high concentrations of NO (Figure 1). The fact that the strain lacking  $\sigma^E$  displayed lowered persistence in mice following experimental infection (Figure 3A) raises the possibility that impaired growth in the host might be due, at least in part, to the inability of this strain to resist NO-stress during infection. This notion was confirmed by our experiments in mice unable to mount an intracellular NO response during infection (iNOS knockout mice). In these animals, the  $\Delta$ sigE strain of *C. pseudotuberculosis* persists much longer than in wild-type mice (Figure 3B); this demonstrated that  $\sigma^E$  is in fact necessary for resistance to nitrosative stress in vivo. Moreover, the significant effect of this mutant on viability of iNOS<sup>-/-</sup> macrophages in culture (Figure 3C), which is expected to be related to the ability of the bacteria to replicate in the intraphagosomal compartment, further corroborates the involvement of  $\sigma^E$  in bacterial survival to reactive nitrogen species.

Since the ECF sigma factor  $\sigma^E$  seems to participate in response to cell envelope stresses in different bacteria, we hypothesized that mutation of its coding gene in *C. pseudotuberculosis* could lead to alterations of this bacterium's extracellular proteome in response to NO-stress. In fact, we could identify differentially regulated exoproteins of *C. pseudotuberculosis*

following NO-treatment. Besides, marked differences were observed between the exoproteomes of the 1002 (wt) and  $\Delta$ sigE strains (Figure 4).

Exportation of proteins involved in metal ion acquisition and redox homeostasis seems to be a primary response of *C. pseudotuberculosis* to biologically relevant concentrations of NO (Table 1). Recent studies on the transcriptional alterations in *M. tuberculosis* and *M. bovis* BCG following treatment by NO or H<sub>2</sub>O<sub>2</sub> also demonstrated that these bacteria up-regulate iron acquisition genes as a specific response to nitrosative and oxidative stresses (Jang et al., 2009; Voskuil et al., 2011). Additional proteins found to be exported in the  $\Delta$ sigE mutant strain of *C. pseudotuberculosis* in response to NO, compared to the 1002 (wt) strain, might be indicative of a compensatory, more general response to stress (Table 1). This is reinforced by the fact that some of these proteins have already been shown to be part of other stimulons in *C. pseudotuberculosis*, regulated by heat shock, osmotic, or acidic stresses (Table 1).

In conclusion, our results demonstrate a role for the ECF sigma factor  $\sigma^E$  in resistance of an intracellular bacterium to nitrosative stress. While deletion of this sigma factor in *C. pseudotuberculosis* did contribute for altering the composition of this bacterium's exoproteome in response to environmental cues, further studies remain necessary to better define SigE-responsive genes that account for the observed nitric oxide-susceptible phenotype of the  $\Delta$ sigE strain.

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