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The impact of manganese on biofilm development of *Bacillus subtilis*

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Bacterial biofilms are dynamic and structurally complex communities, involving cell-to-cell interactions. In recent years, various environmental signals that induce the complex biofilm development of the Gram-positive bacterium *Bacillus subtilis* have been identified. These signalling molecules are often media components or molecules produced by the cells themselves, as well as those of other interacting species. The responses can also be due to depletion of certain molecules in the vicinity of the cells. Extracellular manganese (Mn^{2+}) is essential for proper biofilm development of *B. subtilis*. Mn^{2+} is also a component of practically all laboratory biofilm-promoting media used for *B. subtilis*. Comparison of complex colony biofilms in the presence or absence of supplemented Mn^{2+} using microarray analyses revealed that genes involved in biofilm formation are indeed downregulated in the absence of Mn^{2+}. In addition, Mn^{2+} also affects the transcription of several other genes involved in distinct differentiation pathways of various cellular processes. The effects of Mn^{2+} on other biofilm-related traits like motility, antimicrobial production, stress and sporulation were followed using fluorescent reporter strains. The global transcriptome and morphology studies highlight the importance of Mn^{2+} during biofilm development and provide an overview on the expressional changes in colony biofilms in *B. subtilis*.

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INTRODUCTION

Environmental cues and the spatial distributions of nutrients govern various microbial interactions, i.e. quorum sensing, sporulation and formation of fruiting bodies (He & Bauer, 2014; Kaiser, 2015; Ng & Bassler, 2009; Traxler & Kolter, 2015). The surface attachment and formation of sessile bacterial communities, yielding the so-called biofilms, are also induced by certain concentrations of nutrients and self-produced signalling molecules. Biofilm formation in *Bacillus subtilis* has been extensively studied, and various molecules such as self-secreted surfactin, plant polysaccharides, chlorine dioxides and combinations of glycerol and manganese have been reported to induce a cascade of reactions leading to the production of biofilm matrix components (Beauregard *et al.*, 2013; López & Kolter, 2010; Mhatre *et al.*, 2014; Shemesh *et al.*, 2010; Shemesh & Chai, 2013; Vlamakis *et al.*, 2013). These signal molecules act on distinct histidine sensor kinases and activate Spo0A, which is also required for sporulation through the phospho-relay pathway (Hoch, 1993). Increase in the level of phosphorylated Spo0A (Spo0A~P) results in de-repression of *epsA-O* and *tapA-sipW-tasA* operons that are involved in the synthesis of exopolysaccharide (EPS) and protein (TasA) matrix components, respectively. EPS and TasA promote the attachment of cells to the surfaces or to each other and give rise to complex biofilm structures that help the cells to form pellicles at the air–liquid interface, architecturally complex colonies or submerged aggregates (Branda *et al.*, 2001, 2006). The matured biofilm is protected by a surface-localized amphiphilic protein, BslA, that aids the biofilm surface repellency (Hobley *et al.*, 2013; Kobayashi & Iwano, 2012; Kovács *et al.*, 2012).

Certain levels of Spo0A~P determine the fate of the cell's commitment to biofilm formation or sporulation, and this is coordinated by a phospho-relay mechanism activated by specific membrane-bound kinases, KinA to KinE (López &

Three supplementary figures and one supplementary table are available with the online Supplementary Material.

Kolter, 2010). While the kinases KinA and KinB are mainly important for efficient sporulation, which requires high levels of Spo0A~P (Grimshaw *et al.*, 1998; Jiang *et al.*, 2000), KinC and KinD play a role in the induction of biofilm development with low levels of Spo0A~P (López *et al.*, 2009b; Vlamakis *et al.*, 2013). During the late log phase, cells face many challenges such as depletion of nutrients, oxygen stress, production of toxins and waste molecules and the presence of surfactin-like molecules that cause membrane leakage (Mhatre *et al.*, 2014). These molecules act as environmental cues leading to the activation of kinases that in turn facilitates the transfer of phosphate groups via Spo0F and Spo0B to Spo0A. Several other global transcriptional regulators, like AbrB, DegS/DegU, SlrR, and SinI/SinR, contribute to the complex signalling pathway and interconnected processes aiding the biofilm development of *B. subtilis* (Kovács, 2016; López & Kolter, 2010; Vlamakis *et al.*, 2013). Various biofilm-related developmental processes were identified in *B. subtilis* biofilms involving rugose colony structures (Shemesh & Chai, 2013), reduction of cell motility (Kearns *et al.*, 2005), production of antimicrobial peptides like sporulation-killing factor (Skf) and sporulation delay protein (Sdp) (Gonzalez-Pastor *et al.*, 2003; López *et al.*, 2009b), ESP and TasA secretion or γ-poly-DL-glutamic acid production (Stanley & Lazazzera, 2005).

In this study, we report the impact of manganese (Mn^{2+}) on colony biofilm development and related developmental processes in the *B. subtilis* laboratory strain 168 (hereafter WT168). Manganese ions have diverse functions in living cells: Mn^{2+}-containing metalloenzymes are required for oxidative photosynthesis and various metabolic pathways including the 3-phosphoglycerate mutase, a glycolytic enzyme (Jakubovics & Jenkinson, 2001). Mn^{2+} plays a crucial role in several developmental pathways in bacteria (Helmann, 2014), in particular those belonging to the genus *Bacillus* where reportedly it acts on diverse cell division proteins, including DnaA (Hoover *et al.*, 2010). Moreover, Mn^{2+} is important for the initiation of sporulation by acting as obligate co-factor for the enzyme phosphoglycerate phosphomutase (Vasantha & Freese, 1979). Due to the importance of Mn^{2+} in growth and maintenance of cytoplasmic processes in *Bacilli*, its transport and homeostasis have been actively studied (Helmann, 2014). Suboptimal concentrations of Mn^{2+} result in decreased single- or mixed-species biofilm formation of *Lactobacillus plantarium* (Nozaka *et al.*, 2014). Interestingly, a recent study highlighted that Mn^{2+} is required as a co-factor in the glycerol-mediated robust colony biofilm initiation of *B. subtilis* NCIB 3610 under non-biofilm-inducing conditions (Shemesh & Chai, 2013).

Our transcriptional analysis of colony biofilms grown in the presence or absence of supplemented Mn^{2+} showed that several intertwined processes were downregulated when Mn^{2+} was not supplemented to the medium. The colony structures lacked the white rugose patterns that we describe as chalky patterns, which otherwise are seen in the presence of Mn^{2+}. Testing selected processes that were downregulated in colonies without the addition of $Mn^{2+}$to the medium, we identified *gerR as* important for the formation of the chalky patterns in colonies.

METHODS

Strains and growth media. *B. subtilis* strains used and generated in this study are listed in Table 1. The overnight cultures were grown in LB medium (Lennox broth, Carl Roth, Germany; 1 % tryptone, 0.5 % yeast extract, 0.5 % NaCl). For architecturally complex colonies, 2× Schaeffer's sporulation medium containing 0.1 % glucose (2× SG) and 1.5 % agar was used (Kobayashi, 2007; Kovács & Kuipers, 2011), containing 0.1 mM of $MnCl_2$ unless omitted. The colony biofilms were also assayed on LB medium containing 0.1 mM $MnCl_2$ and 0.1 % glucose or 1 % glycerol with 1.5 % agar (Shemesh & Chai, 2013), or MSgg medium as described earlier (Branda *et al.*, 2001). For submerged biofilms, biofilm growth medium (BGM) consisting of LB, supplemented with 0.15 M $(NH_4)_2SO_4$, 100 mM $K_xH_yPO_4$ (pH 7), 34 mM Na-citrate, 1 mM $MgSO_4$, 0.1 % glucose and 0.1mM $MnCl_2$, was used as described previously (Hamon & Lazazzera, 2001). *Escherichia coli* strain (MC1061) used for molecular cloning was grown at 37 °C in LB medium. Antibiotics were included wherever appropriate at the following concentrations: 7.5 μg ml^{-1} of tetracycline, 5 μg ml^{-1} of kanamycin, 100 μg ml^{-1} of spectinomycin, 5 μg ml^{-1} of chloramphenicol, 100 μg ml^{-1} of ampicillin and 12.5 μg ml^{-1} of lincomycin, together with 1 μg ml^{-1} of erythromycin (for Mls resistance).

Colony biofilm and imaging. Overnight-grown *B. subtilis* cultures were inoculated at 1 % concentration in 2× SG medium in 24-well plates for pellicle biofilms or 2 μl was spotted on 2× SG, MSgg or LB medium (with $MnCl_2$ and glucose or glycerol) agar plates for observing complex colonies, and incubated at 30 °C for 3 days. Both pellicle and colony biofilms were observed after 3 days and images were taken using an AxioZoom V16 microscope equipped with an AxioCam MRm monochrome camera (Carl Zeiss Microscopy GmbH, Jena, Germany).

Strain constructions. The mutants and the reporter gene constructs used in this study were introduced into parental *B. subtilis* strain 168 (WT168) by transforming genomic DNA extracted from various strains listed in Table 1 using the 2-step transformation protocol (Anagnostopoulos & Spizizen, 1961). To construct the P*sboA-gfp* fusion, the *sboA* promoter region was PCR amplified using primers oEM3 and oEM4 (Table 2), digested with *Eco*RI and *Nhe*I enzymes, ligated into the corresponding sites of pGFP-rrnB (Veening *et al.*, 2009), and transformed into *E. coli*. The resulting plasmid, pTB65 was then transferred into *B. subtilis* WT168 and double recombination into the *amyE* locus was verified. For the construction of mutants Δ*yitB* and Δ*ywoF*, the upstream and downstream regions of the genes were PCR amplified (see Table 2 for primers sequences) and sequentially cloned into the *Kpn*I-*Sal*I and *Bam*HI-*Xba*I sites of pTB120 (Hölscher *et al.*, 2015). The resulting plasmids pTB591 and pTB592 were then transferred into *B. subtilis* and deletions were verified by PCR. The sequences of all newly generated plasmids were verified by DNA sequencing at GATC Biotech (Cologne, Germany). Transformants obtained using genomic DNA of mutant strains with antibiotic cassettes were verified using PCR with primers indicated in Table 2.

Transcriptome analysis. Colonies were harvested after 3 days of growth on 2× SG medium with or in the absence of Mn^{2+} supplementation. RNA extraction was performed with the Macaloid/Roche protocol (Kovács & Kuipers, 2011), with two additional steps of phenol–chloroform washing. RNA concentration and purity were assessed using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific). RNA samples were reverse transcribed into cDNA using the Superscript III reverse transcriptase kit (Invitrogen, Carlsbad, USA) and labelled

Table 1. Strains and plasmids used

*Km^R, kanamycin resistance; Cm^R, chloramphenicol resistance; Tet^R, tetracycline resistance; $Spec^R$, spectinomycin resistance, Amp^R, ampicillin resistance; Mls^R, macrolide resistance (erythromycin+lincomycin).

Strain	Genotype*	Reference or source
B. subtilis		
168 1A700	*trpC2*	Laboratory collection (Kovács & Kuipers, 2011)
NCIB 3610	undomesticated wild type	Branda *et al.* (2001)
GC260	PY79 Δ*gerR*:: Km^R	Cangiano *et al.* (2010)
TB61	168 Δ*gerR*:: Km^R	This study
DL227	NCIB 3610 Δ*kinC*:: Mls^R	López *et al.* (2009a)
DL153	NCIB 3610 Δ*kinD*:: Tet^R	
TB148	168 Δ*kinC*:: Mls^R	This study
TB149	168 Δ*kinC*:: Mls^RΔ*kinC*:: Tet^R	This study
TB126	168 Δ*kinD*:: Tet^R	This study
TB417	168 Δ*gerR*:: Km^R, *amyE*:: *gerR* $Spec^R$	This study
TB65	168 *amyE*:: P_{sboA}-*gfp* Cm^R	This study
hag-gfp	168 P_{hag}-*gfp* Cm^R	Veening *et al.* (2008)
IIA-gfp	168 $P_{spoIIAA}$-*gfp* Cm^R	Veening *et al.* (2005)
ΔspoIIAC	168 Δ*sigF*:: Km^R	Veening *et al.* (2006a)
DL821	NCIB 3610 *lacA*:: P_{tapA}-*yfp* Mls^R	López *et al.* (2009c)
TB64	168 *lacA*:: P_{tapA}-*yfp* Mls^R	This study
TB49	168 *amyE*:: Phyperspank- *gfp* Cm^R	van Gestel *et al.* (2014)
RL52	Δ*cotC*:: Km^R *trpC2*	Resnekov *et al.* (1995)
TB416	168 Δ*cotC*:: Km^R	This study
TB553	168 Δ*yitB*:: Km^R	This study
TB554	168 Δ*ywoF*:: Km^R	This study
E. coli		
MC1061	F^- Δ(*ara-leu*)769 $[araD139]_{B/r}$ Δ(*codB-lacI*)3 *galK*16 *galE*15 λ^- e14$^-$ *mcrA*0 *relA*1 *rpsL*150(Str^R) *spoT*1 *mcrB*1 *hsdR*2(r^-m^+)	Casadaban *et al.* (1980)

Plasmid	Genotype	Reference
pGFP-rrnB	*amyE*, P_{rrnB}-*gfp*+, Cm^R, $Spec^R$, Amp^R	Veening *et al.* (2009)
pTB65	P_{sboA} cloned into pGFP-rrnB, Amp^R, Cm^R	This study
pWK-Sp	*amyE*, Amp^R, $Spec^R$ derived from pDG1727	Veening *et al.* (2006b)
pTB417	*gerR* cloned into pWK-Sp, Amp^R, $Spec^R$	This study
pTB120	Nm^R cassette cloned into pBluescript SK +	Hölscher *et al.* (2015)
pTB591	Nm^R cassette with upstream and downstream regions of *B. subtilis yitB*	This study
pTB592	Nm^R cassette with upstream and downstream regions of *B. subtilis ywoF*	This study

with Cy3 or Cy5 monoreactive dye (GE Healthcare, Amersham, The Netherlands). Labelled and purified cDNA samples (Nucleospin Extract II, Biokè, Leiden, The Netherlands) were hybridized in Ambion Slidehyb #1 buffer (Ambion Europe Ltd) at 48 °C for 16 h. The DNA-microarrays were constructed as described previously (Van Hijum *et al.*, 2003). Briefly, specific oligonucleotides for all 4.107 open reading frames of WT168 were spotted in duplicate onto aldehyde-coated slides (Cell Associates) and further handled using standard protocols for aldehyde slides. Slide spotting, slide treatment after spotting and slide quality control were done as earlier (Kuipers *et al.*, 2002). After hybridization, slides were washed for 5 min in 2× SSC with 0.5 % SDS, 2 times 5 min in 1× SSC with 0.25 % SDS, 5 min in 1× SSC 0.1 % SDS, dried by centrifugation (2 min, 2000 rpm) and scanned in GenePix 4200AL (Axon Instruments, CA, USA). Fluorescent signals were quantified using ArrayPro 4.5 (Media Cybernetics Inc., Silver Spring, MD, USA) and further processed and normalized with MicroPrep (Van Hijum *et al.*, 2003). CyberT (Baldi & Long, 2001) was used to perform statistical analysis. Genes with a Bayes *P*-value of $\leq 1.0\times10^{-4}$ were considered significantly affected. Microarray data have been deposited in the Gene Expression Omnibus database (Accession No. GSE61232).

Fluorescence measurement. *B. subtilis* strains harbouring given promoter fusion constructs were grown overnight in LB medium and inoculated at 1 % concentration in 2× SG medium with or without supplementation of Mn^{2+}, into 96-well plates and incubated at 30 °C under continuous shaking using a microplate reader (Infinite 200 PRO,

Table 2. Oligonucleotides used

Oligo name	Sequence	Target locus or marker
oEM3	5′-GTGGTGCGGAATTCGATGAC-3′	*sboA* promoter
oEM4	5′-CGAGGCTAGCGACAGCTTTTTTCATAATTG-3′	*sboA* promoter
oEM25	5′-TGGACAGTTGCGGATGTACTTCAG-3′	*km* marker check
oEM28	5′-TTCAGCCACTGCATTTCC-3′	*spec* marker check
oEM33	5′-TCCGCTGTCAACGATACTTC-3′	*gerR* check primer
oEM34	5′-TGAAGCTGATGCTGCTTCAC-3′	*gerR* check primer
oEM35	5′-GATTGGCCGCTTACACATGG-3′	*neo* marker check
oEM44	5′- ATGGATCCTGCGGATCATACAACGG-3′	*gerR* complementation
oEM45	5′- ATTCTAGAGATGACACCGGGAGAGTCTGC-3′	*gerR* complementation
oEM6	5′- GCAGGTCGACAACGAAAGCGAGGATGACAG-3′	*ywoF* upstream
oEM46	5′- ATGGTACCGCGTTCGGAGAGCCGATTTG-3′	*ywoF* upstream
oEM47	5′- ATGGATCCTGATTCACCTGCGCGGCCTG-3′	*ywoF* downstream
oEM48	5′- ATGCGGCCGCCAGCCTTTGCGCTGTTTATG-3′	*ywoF* downstream
oEM13	5′- CGAGGTCGACAACACATCGAGCTCATC-3′	*yitB* upstream
oEM49	5′- ATGGTACCCGCAGCCGTTACCACATTTG-3′	*yitB* upstream
oEM50	5′- ATGGATCCGATGAGAGGGCTGGAAGATG-3′	*yitB* downstream
oEM51	5′- GCTCTAGATCTCCGACAAGTGGGTGAAG-3′	*yitB* downstream
oEM5	5′- TGATATTGCTGGAGGATTGG-3′	*ywoF* check primer
oEM8	5′- ATTGCCGCTGATATTATTCC-3′	*ywoF* check primer
oEM21	5′- TTTGGGCGTCGTATGTTCC-3′	*yitB* check primer
oEM22	5′- CGGCTATCGCATCTTTG-3′	*yitB* check primer

Switzerland). i-Control software was used to monitor the fluorescence (gain used: 40) with GFP [excitation at 485 nm (20 nm width) and emission at 535 nm (10 nm width)] or YFP filter sets (excitation at 495 nm [10 nm width] and emission at 540 nm [25 nm width]) every 15 min for 20 h. Emission from medium-only control wells was subtracted from the data and normalized to the optical density of the culture measured at 595 nm. The graphs were plotted using OriginLab 2015 software.

Submerged biofilm assay. The fluorescent-labelled cultures of WT168 (TB48) (van Gestel *et al.*, 2014) were pre-grown in LB medium and transferred to BGM medium to obtain overnight cultures at 37 °C under well-shaken conditions. Next 2 µl of overnight culture was added to 200 µl of BGM medium with and without added Mn^{2+} in a Falcon 96-well black flat-bottom TC-treated Microplate (Corning Life Science, USA) and incubated at 30 °C under static conditions. After 6 h, the samples were mixed thoroughly and the medium was changed every 12 h. After 24 h, the biofilms were observed using a Zeiss LSM780 confocal laser-scanning microscope fitted with a 488 nm laser and an EC Plan-Neofluar 10× differential interference contrast M27 objective (Carl Zeiss Microscopy GmbH, Jena, Germany). Tile scan of the entire well was taken and the images were captured with Zen Black software (Carl Zeiss Microscopy GmbH, Jena, Germany).

Sporulation assay. The 3-day-old complex colonies of WT168, Δ*gerR* and Δ*sigF* from 2×SG agar plates with and without $MnCl_2$ were harvested and suspended in 1 ml of 2× SG medium. The colony clumps were sonicated (2×12 pulses of 1 s with 30 % amplitude; Ultrasonic Processor VCX-130, Zinsser Analytics, Frankfurt am Main, Germany). Cells were then diluted and plated on LB medium supplemented with 1.5 % agar to determine the viable colony count. Samples were then incubated at 80 °C for 20 mins and plated on LB agar medium to record the spore count. Sporulation efficiency was calculated by dividing the spore number by the viable cell number (Ireton *et al.*, 1994). The experiment was performed twice on independent occasions with four replicates each time.

RESULTS

Mn^{2+} influences robust pellicle formation and rugose colony structure

To examine the importance of Mn^{2+} on the biofilm-proficient laboratory strain WT168, pellicle and colony structures were studied with or without addition of Mn^{2+} in the media. 2× SG medium was utilized, as this medium efficiently promotes the formation of complex biofilm structures of *B. subtilis* WT168 compared to the previously used MSgg minimal medium for wild isolates of *B. subtilis* (Kovács & Kuipers, 2011). No pellicle formation was observed in the absence of Mn^{2+} addition to the 2× SG medium (Fig. 1a). Intrigued by this observation, we also tested the undomesticated strain NCIB 3610 in MSgg medium in the absence of $MnCl_2$. The pellicle formed in the absence of Mn^{2+} was weak and fragile (Fig. 1a). The inability of *B. subtilis* cells to form robust pellicles in the absence of Mn^{2+} suggests that it is essential for the proper induction of this developmental pathway. Furthermore, omitting any other ion component of the 2×SG medium, other than Mn^{2+}, had less or no effect on pellicle formation (data not shown).

Furthermore, the colony biofilms showed distinguishable patterns on agar plates with and without added Mn^{2+}. Colony biofilms are rugose, with vein-like structures projecting from the agar surface and bundles of cells chained together in a parallel pattern (Branda *et al.*, 2001; Cairns *et al.*, 2014). A close examination of colony biofilms in

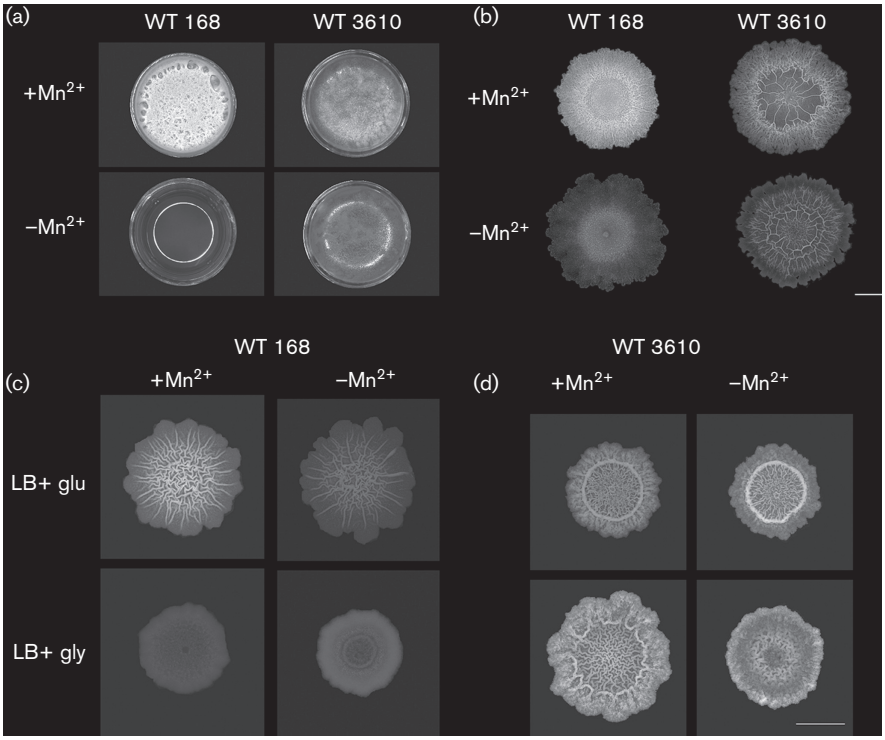

Fig. 1. Effect of Mn^{2+} on *B. subtilis* biofilms. Air-medium pellicle (a) and complex colony structures of laboratory (WT168) (b and c) and undomesticated (NCIB 3610) (d) *B. subtilis* are shown in various media with or without supplemented Mn^{2+}. The colony structures were assayed in Mn^{2+}-proficient or -deficient conditions on 2× SG (b) or LB medium with glucose (0.1 %) or glycerol (1 %) (c). The scale bars at the lower right corner in panels (b) and (c) represent 5 mm.

WT168 reveals concentric white chalky patterns that become less and less evident at the edges of the colony. These chalky patterns were absent in colonies grown on 2× SG medium without addition of Mn^{2+}, making the colonies appear pale (Fig. 1b) but not as shiny as strains lacking EPS production [e.g. *epsG* mutant described in Kovács & Kuipers (2011)]. Similar observations were made when colonies were grown on LB agar medium supplemented with either glucose or glycerol in the absence and presence of added Mn^{2+}(Fig. 1c). In the absence of added Mn^{2+} in the MSgg medium, NCIB 3610 showed pale colonies lacking the white chalky patterns (Fig. S1, available in the online Supplementary Material).

Transcriptional profiling of biofilm colonies grown with different Mn^{2+} concentrations

We were interested to see whether other processes within colony biofilms are also altered by the lack of Mn^{2+}; in other words, whether Mn^{2+} has a global and important effect in biofilm development. In order to pursue this, colony biofilms were harvested from 2× SG medium containing plates with and without supplemented Mn^{2+}. Whole-genome DNA-microarray experiments performed on these colonies revealed significant up- and down-regulation of transcription of 133 and 305 genes, respectively (Table S1, available in the online Supplementary Material), by at least fourfold (Bayesian *P* value $<10^{-4}$). The list of differentially transcribed genes was extensive, involving about 10 % of the genes on the genome of *B. subtilis*, and included various basic metabolic processes as well. Therefore, Mn^{2+} may play a pleiotropic role in the colony biofilm development of *B. subtilis*. As expected, genes related to Mn^{2+} transport (*mntA, mntB, mntC* and *mntH*) were upregulated in the absence of supplemented Mn^{2+} in the medium (Table S1). Microarray results clearly showed that genes related to biofilm formation (e.g. *epsA-O* and *tapA-sipW-tasA* operons) and sporulation (including numerous *spo, cot, sps, ssp, ger* genes) are downregulated when Mn^{2+} is not supplied in the medium (Table S1). Interestingly, at the same time that reduced biofilm formation took place, genes related to flagellar motility were transcribed at a higher level in colony biofilms grown on Mn^{2+}-depleted medium. Transcriptions of various other genes related to antimicrobial peptide production (e.g. *sboA, skfA-E* and *sdpA-D*), sporulation (*spoIIA, gerR*), iron uptake in cells (*ymfD)*, sulfate reduction (*yitB)* and a few genes of unknown function (*ywoF*) were downregulated when Mn^{2+} was not supplied in the 2× SG medium.

Role of Mn^{2+} in sporulation results in the presence of chalky patterns

Previous studies on sporulation in planktonic cultures have demonstrated that Mn^{2+} plays a role in activating the key enzymes needed for spore formation in *B. subtilis*. These studies report that Mn^{2+} affects the initiation of sporulation and is a critical component of the media used for these studies during the early growth phases before the spores appear, as subsequent addition of Mn^{2+} salts did not facilitate spore formation (Charney *et al.*, 1951). Later studies linked Mn^{2+} to phosphoglycerate phosphomutase, an enzyme needed for sporulation in the basic sporulation media (Vasantha & Freese, 1979). To confirm whether the chalky pattern of complex colonies is caused by the presence of spores and not just cell debris (Webb *et al.*, 2003), we observed the colony biofilms of a *sigF* mutant that had a sporulation defect. The complex colony of the *sigF* mutant lacks the chalky pattern in the absence of Mn^{2+}, similar to WT168 (Fig. 2a), while the colony structure of the *sigF* strain is slightly altered in the absence of added Mn^{2+} in comparison to WT168. Thus, the sporulation pattern in colonies is affected by the absence of Mn^{2+}, and spores are important in regard to the appearance of chalky structures.

Transcriptome analysis done on colonies harvested from the medium with and without supplemented Mn^{2+} revealed that the sporulation process and the genes related to the formation of spore coat proteins (*gerPB*, *gerPD* and *gerPE*) are downregulated in the absence of Mn^{2+}. In our screening for mutants with altered biofilm development (see description of the screen in the following text) that phenotypically lacks the chalky patterns, we found the *gerR* mutant, which showed reduction in chalky patterns even in the presence of Mn^{2+}. In the absence of Mn^{2+}, the chalky pattern was further reduced (Fig. 2a). The structures were restored partially after reintroducing the *gerR* gene into the *amyE* locus of the *gerR* mutant strain. Sporulation efficiency in complex colonies was also quantified by enumerating germinated spores after the heat treatment. We observed that the spore count was reduced in the absence of Mn^{2+} in the growth medium in both WT168 and *gerR* (Fig. 2b). The *sigF* mutant colonies were used as control, as this mutant was reported to be defective in spore formation (Yudkin, 1987).

It was previously shown that although GerR does not appear to play a direct role in the expression of genes, many σ^E-and SpoIIID-controlled genes are downregulated in its absence (Eichenberger *et al.*, 2004). GerR also plays a role in spore coat development (Kuwana *et al.*, 2005) and hence the alterations in spore properties might explain the absence of the white rugose structures and the pale colony appearance. However, the colony morphology of a *cotC* mutant exhibiting a defect in the outer spore coat protein shows no phenotypic differences in colony structure (Fig. S2).

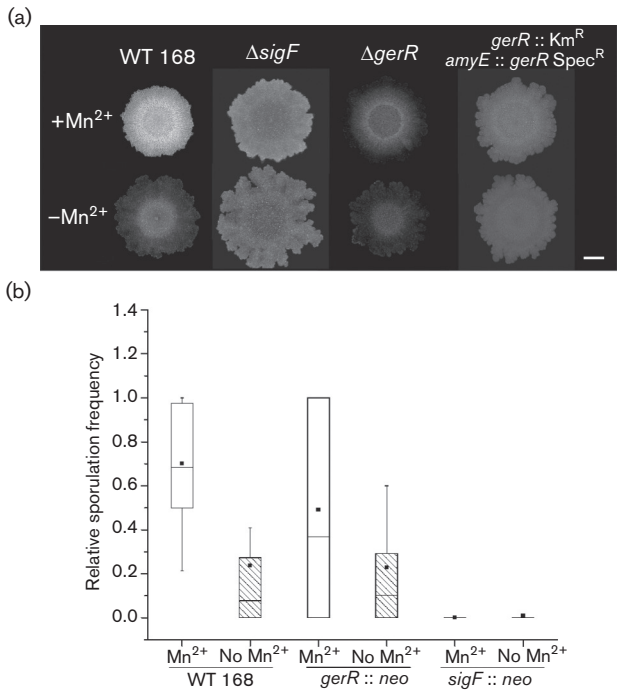

Fig. 2. Mutations in *gerR* and *sigF* affect rugose patterns in *B. subtilis* colonies. (a) Colony structures of mutant strains were tested in the presence and absence of supplemented Mn^{2+}. The scale bar at the lower right corner represents 5 mm. (b) The relative spore count is shown in WT168, *gerR* and *sigF* colony biofilms in the presence or absence of Mn^{2+} supplementation.

Mn^{2+} affects the surface coverage of submerged biofilms of *B. subtilis*

To determine whether the presence of Mn^{2+} is also essential for the formation of submerged biofilms, WT168 cells were inoculated on an optical microtitre-plate using medium described previously for submerged biofilm formation in *B. subtilis* (Hamon & Lazazzera, 2001). The bottom surface area of the wells containing Mn^{2+}-supplemented medium was entirely or mostly covered with biofilms while in the wells with Mn^{2+}-limited medium, the surface coverage by cells was reduced (Fig. 3). This suggests that Mn^{2+} is required for cell attachment and/or biofilm development on the submerged surface of the medium.

The presence or absence of Mn^{2+} affects processes including motility, biofilm matrix production, sporulation and antimicrobial peptide production

To validate the microarray experiments performed on biofilms grown at different Mn^{2+} levels, promoter fusion constructs were used to follow the expression of genes related to motility ($P_{hag\text{-}gfp}$) (Veening *et al.*, 2008), biofilm matrix protein production ($P_{tapA\text{-}yfp}$) (López *et al.*, 2009c), sporulation ($P_{spoIIE\text{-}gfp}$) (Veening *et al.*, 2005) and antimicrobial peptide production ($P_{sboA\text{-}gfp}$). The expression of these genes was followed in microtitre plates for 20 h using 2×SG medium with and without the addition of Mn^{2+} (Fig. 4).

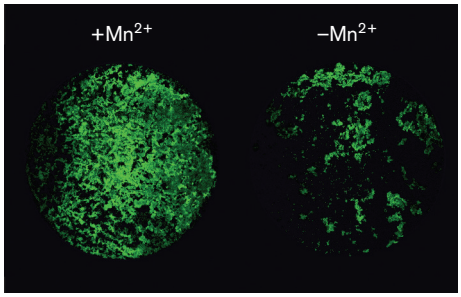

Fig. 3. Effect of Mn^{2+} on submerged biofilms. Submerged biofilms were grown for 24 h at 30 °C and the images were taken using a Zeiss CLSM tile scan at 10× magnification. Representative wells are shown for both conditions.

Under these well-mixed growth conditions, expression of the *hag* gene was similar at the start of growth, but showed a diminished expression in Mn^{2+}-supplemented medium during the stationary phase of growth. The P_{tapA}-*yfp* and P_{spoIIE}-*gfp* constructs showed gradual but linear increase in expression in the cells grown in the presence of Mn^{2+} and were at their peak during the stationary phase. The expression profile of the *sboA* gene, involved in the production of the cyclic bacteriocin subtilosin (Zheng *et al.*, 1999), showed a linear increase, with a peak at the 12 hour of growth followed by reduction in the stationary phase. These experiments clearly showed the Mn^{2+}-dependent expression of selected genes not only in colony biofilms, but also in planktonic cells grown in well-agitated, but otherwise biofilm-inducing, medium. We investigated whether the absence of Mn^{2+} affects the growth of WT168 in liquid or solid 2× SG medium. The fact that the colony sizes in the presence and absence of Mn^{2+} were comparable suggests no severe growth defect. The growth rates of WT168 under planktonic conditions with or without Mn^{2+} addition were similar, while the final yield in the absence of supplemented Mn^{2+} was slightly lower (Fig. S3).

Mn^{2+} affects colony biofilm development in *B. subtilis* WT168 independently of KinC and KinD histidine kinases

The effects of signalling molecules that induce biofilm formation and sporulation in *B. subtilis* are often facilitated via membrane-bound histidine kinases that increase the level of phosphorylated Spo0A through a phospho-relay (Mhatre *et al.*, 2014; Vlamakis *et al.*, 2013). The presence of molecules such as surfactin, nystatin and their derivatives was suggested to induce pore formation in the cell membrane leading to potassium leakage, which activates the signalling domain of KinC (Gonzalez-Pastor *et al.*, 2003; Lopez *et al.*, 2009a, 2010); however, the impact of surfactin on KinC-mediated activation is debated and might be condition- or strain-specific (Devi *et al.*, 2015; López, 2015). In addition, plant polysaccharides and secreted sugars activate KinD (Beauregard *et al.*, 2013; Chen *et al.*, 2012). Mn^{2+} was suggested to act as a co-factor mediating phosphate transfer in response to the presence of glycerol under biofilm non-promoting conditions (Shemesh & Chai, 2013). Therefore, the effect of inactivating *kinC* or *kinD* genes was assayed on colony biofilm development with and without Mn^{2+} addition. While mutations in either or both *kinC* and *kinD* resulted in altered colony architecture, the biofilm colonies on 2× SG medium with or without addition of Mn^{2+} still differed, suggesting that Mn^{2+} influences colony development, at least partially, independent of the KinC and KinD kinases (Fig. 5).

Testing the impact of selected Mn^{2+}-influenced processes on colony biofilm development

The microarray analysis showed that the transcription of several genes was reduced in the medium depleted in Mn^{2+}, including that of the genes related to biofilm formation. Could any of these genes be involved in rugose colony formation? To identify additional genes that are required for the development of architecturally complex colonies, we selected the so-called '*y*' genes that were annotated as genes of unknown function when the genome sequence was published (Kunst *et al.*, 1997). The library of insertion mutants (Bacillus functional analysis, BFA) that was created previously in various European laboratories and contains around 1200 mutants of the ~2600 '*y*' genes (Kobayashi *et al.*, 2003) was used. The genes that had an altered expression in this transcriptome study, with respect to supplemented Mn^{2+} levels, were selected and mutants were tested for colony formation on 2× SG. From this screen, several BFA mutants were identified with potentially altered colony biofilm structure. As these BFA strains were created in various laboratories in Europe, genomic DNA was extracted and transformed into WT168 to circumvent the effects that might have originated from the usage of genetically distinct parental strains. Since most BFA mutants used for screening were constructed by single recombinant events, selected downregulated genes (*yitB, ywoF, gerR*) were chosen to create double recombinant knockouts and to check the colony morphology. As presented earlier, the *gerR* mutant had altered colony morphology. However, the other mutants (*yitB* and *ywoF*) showed no altered colony morphology under colony biofilm conditions (Fig. S2).

DISCUSSION

In this study, the role of an essential and abundantly occurring element, Mn^{2+}, is examined during biofilm formation and during the processes that orchestrate *B. subtilis* biofilms. Most media used in studying biofilm formation in *B. subtilis* include Mn^{2+} salts as one of their minor, but important, components (Yudkin, 1987). Recently, glycerol in combination with Mn^{2+} under biofilm non-promoting conditions, i.e. LB medium, was reported to stimulate formation of structured colony biofilms in *B. subtilis* NCIB 3610. (Shemesh & Chai, 2013). The laboratory strain of *B. subtilis*,

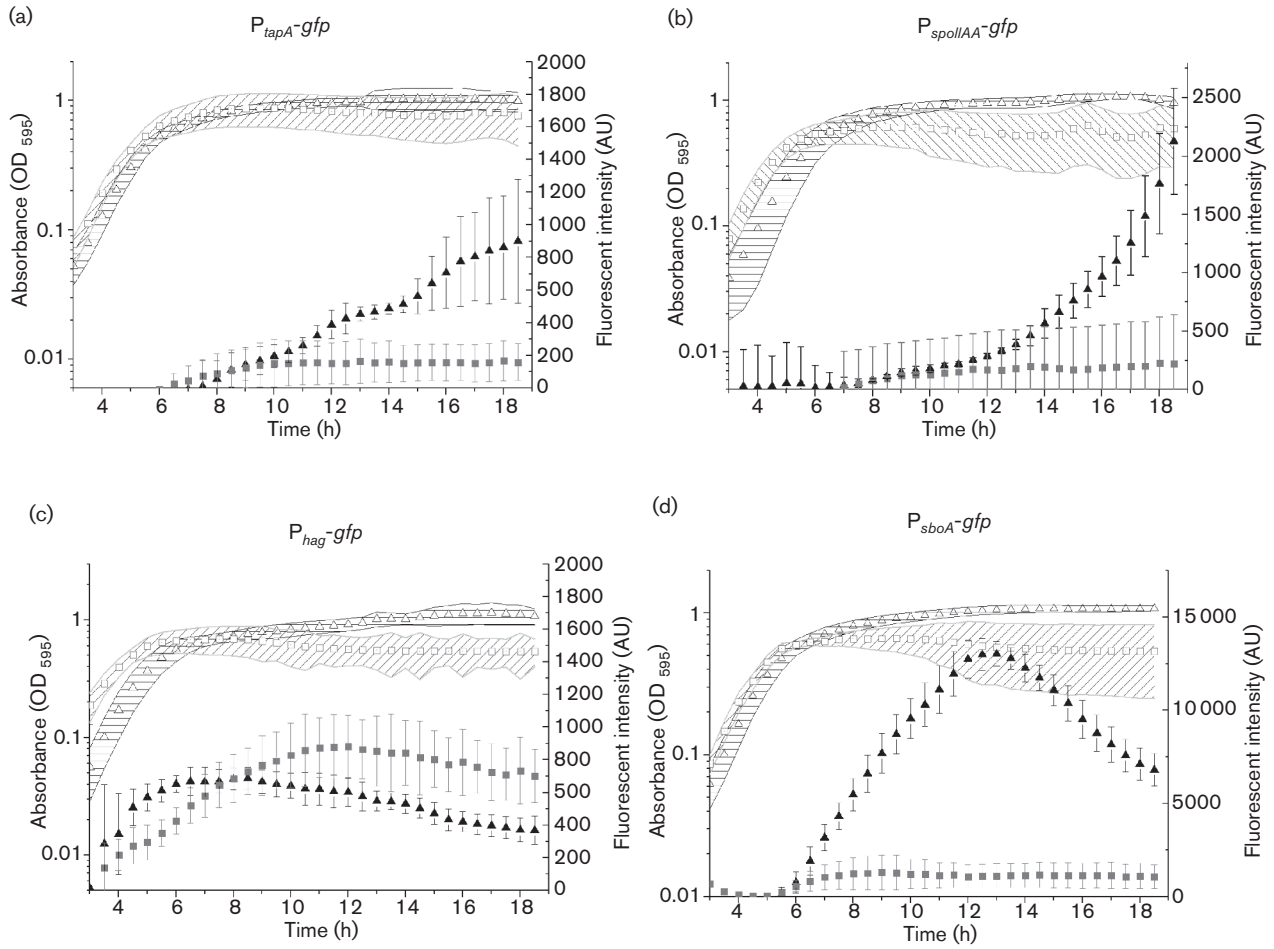

Fig. 4. Expression levels of genes playing a role during onset of biofilm formation. Expression levels of *tapA* (a), *spoIIAA* (b), *hagA* (c) and *sboA* (d) genes were measured in TECAN during agitated growth with (filled triangles) and without (filled squares) supplementation of Mn^{2+} in the 2×SG medium. The optical densities of the cultures are shown with (open triangles) or without (open squares) supplementation of Mn^{2+} in the medium.

WT168, grows better in the presence of glucose than glycerol, and Mn^{2+} itself has a major impact on its characteristic white rugose structures that we describe as chalky patterns. Moreover, omitting Mn^{2+} salts in the rich medium 2× SG stalls pellicle formation. It is clear that Mn^{2+}-dependent processes cause the chalky structures and also aid in pellicle formation. We proceeded to perform DNA microarray studies on colonies grown in the presence and absence of supplemented Mn^{2+} to identify the up- and downregulated processes in *B. subtilis* WT168 during biofilm development.

The list of downregulated processes includes biofilm formation (*epsA-O, tapA-sipW-tasA* operons), sporulation genes and regulators (*spo, sps, spp, cot, ger* genes), amino acids and sugar transporters (*yveA, yocN*), surfactin production, cannibalism and antimicrobial peptide production (*sboA, sdpA, skf*). Importantly, the transcriptions of several differentially expressed genes, including the sporulation-related genes, biofilm development genes and the *skf* and *sdp* operons, are also regulated by different levels of phosphorylated Spo0A (Fujita *et al.*, 2005), which suggests that Spo0A~P levels might be altered in the absence of Mn^{2+}. Notably, a few processes were also upregulated, such as ion transporters, especially Mn^{2+} (*mntABC, mntH*), Zn^{2+} (*zosA*) and Fe-S clusters (*sufC*), motility (*hag*), cell shape and elongation (*rodA*) and the regulators of stress and phospho-relay pathways (*yaaT*). Our screening of the BFA single recombinant mutant library to identify novel biofilm-related genes showed an overlap with the differentially regulated genes. However, clean deletion of some of these candidate genes (constructing double recombinant deletions), other than *gerR*, provided no altered biofilm phenotypes suggesting that the identified biofilm mutants were false negatives possibly because of the construction method of the BFA mutants or through using a different 168 derivate for library construction.

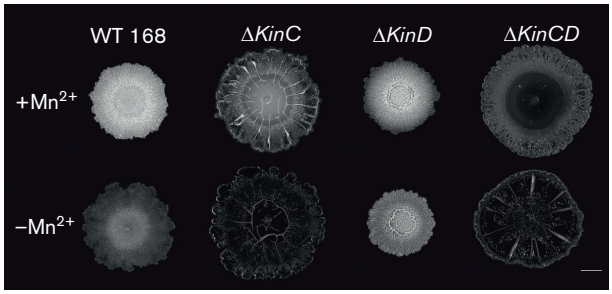

Fig. 5. Mn^{2+}effect on colony structures is independent of the membrane-bound histidine kinases, KinC and KinD. The colony structures of WT168, *kinC* and *kinD* single and double mutants are shown with and without supplementary Mn^{2+} in the 2× SG medium. The scale bar at the lower right corner represents 5 mm.

Previous studies have highlighted the crucial role of Mn^{2+} during initiation and germination of spores in many bacteria belonging to the *Bacillus* genus (Vasantha & Freese, 1979). We propose that alteration in sporulation properties of the colony biofilms explains the absence of the chalky, white pattern observed in mature colony biofilms of *B. subtilis* in the absence of Mn^{2+}. The sporulation mutant *sigF* and late-sporulation regulator *gerR* show either absence of or reduced chalky structures, respectively, in 2× SG medium. Using a different strain (NCIB 3610) and a different medium (MSgg), Branda and colleagues (2001) showed that the absence of spores did not affect the formation of aerial structures, and the colonies formed by a *sigF* mutant were similarly rugose to the wild-type strain. This controversy might be due to the differences between the strains or media used.

GerR regulates the expression of late-sporulation genes, mainly the sigma factors and the spore-coat proteins (Cangiano *et al.*, 2010; Eichenberger *et al.*, 2004). In the absence of *gerR*, cells are still able to form spores while the sporulation frequency is reduced in the absence of Mn^{2+}. Thus, our results clearly suggest that both early and late sporulation-related processes are essential for the chalky patterns seen in colony biofilms of *B. subtilis* WT168. During biofilm development of *B. subtilis*, many different cell-types coexist and the population is phenotypically diverse. Similarly, sporulation is a heterogeneous process as only a sub-population of cells activates genes related to this process. This might explain why the chalky patterns are not present uniformly in the colony and are mostly evident in the periphery around the centre of the colony.

We additionally screened mutants with deletion of genes that were downregulated in our microarray experiment; however, no additional Mn^{2+}-dependent process was identified as being responsible for colony morphology. Monitoring the gene expression of certain biofilm-related processes, e.g. matrix production, cannibalism via the Skf/Sdp proteins, and sporulation, confirmed that Mn^{2+} plays a role in their expression. Divalent anions are known to play a role in regulatory and metabolic networks; however, the effect of Mn^{2+} is more pronounced under biofilm-inducing conditions in *B. subtilis.* The straightforward mechanism by which Mn^{2+} induces biofilm-related processes in *B. subtilis* WT168 is unclear [i.e. the previously suggested *kinD* mutant (Shemesh & Chai, 2013) has different properties in the presence or absence of Mn^{2+}], but in addition to a putative direct effect, it might be indirectly affected by additional biofilm-related processes.

In this study, we defined the colony characters on the basis of concentric chalky patterns that are observed in *B. subtilis* WT168 when grown on 2×SG medium in the presence of Mn^{2+}. This study highlights the importance of Mn^{2+} during biofilm development and provides information to help identify genes with Mn^{2+}-dependent expression that could be related to biofilm formation.

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