Control of chitin and N-acetylglucosamine utilization in *Saccharopolyspora erythraea*

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Chitin degradation and subsequent N-acetylglucosamine (GlcNAc) catabolism is thought to be a common trait of a large majority of actinomycetes. Utilization of aminosugars had been poorly investigated outside the model strain *Streptomyces coelicolor* A3(2), and we examined here the genetic setting of the erythromycin producer *Saccharopolyspora erythraea* for GlcNAc and chitin utilization, as well as the transcriptional control thereof. *Sacch. erythraea* efficiently utilize GlcNAc most likely via the phosphotransferase system (PTSGlcNAc); however, this strain is not able to grow when chitin or N,N’-diacetylchitobiose [(GlcNAc)2] is the sole nutrient source, despite a predicted extensive chitinolytic system (chi genes). The inability of *Sacch. erythraea* to utilize chitin and (GlcNAc)2 is probably because of the loss of genes encoding the DasABC transporter for (GlcNAc)2 import, and genes for intracellular degradation of (GlcNAc)2 by β-N-acetylglucosaminidases. Transcription analyses revealed that in *Sacch. erythraea* all putative chi and GlcNAc utilization genes are repressed by DasR, whereas in *Strep. coelicolor* DasR displayed either activating or repressing functions whether it targets genes involved in the polymer degradation or genes for GlcNAc dimer and monomer utilization, respectively. A transcriptomic analysis further showed that GlcNAc not only activates the transcription of GlcNAc catabolism genes but also activates chi gene expression, as opposed to the previously reported GlcNAc-mediated catabolite repression in *Strep. coelicolor*. Finally, synteny exploration revealed an identical genetic background for chitin utilization in other rare actinomycetes, which suggests that screening procedures that used only the chitin-based protocol for selective isolation of antibiotic-producing actinomycetes could have missed the isolation of many industrially promising strains.

INTRODUCTION

Filamentous soil-dwelling actinomycetes are Gram-positive bacteria that participate in the recycling of organic matter through their remarkably diversified enzymic machinery specialized in carbohydrate utilization (Bertram et al., 2004; Hodgson, 2000). Chitin, the β-1,4-linked polymer of N-acetylg glucosamine (GlcNAc), is the second most abundant polysaccharide on Earth, and mainly originates from the cuticle of arthropods and the cell wall of filamentous fungi. The ability to metabolize chitin is a competitive advantage for soil-dwelling micro-organisms. Indeed, soils are generally carbon-rich and nitrogen-poor environments (Hodgson, 2000); GlcNAc-containing polymers are a rich source of both these essential nutrients.

The metabolic importance of GlcNAc is evident from the degradation products of its catabolism, i.e. (i) ammonia, the preferred nitrogen source for micro-organisms, (ii) fructose-6-phosphate (Fru-6P), which directly enters glycolysis, and (iii) acetate, which, when converted into acetyl-CoA, directly feeds a plethora of biological processes. Additionally, GlcNAc has been shown to be a signalling molecule involved in several cellular processes such as: (i)
morphogenesis in Candida albicans (Gilmore et al., 2013; Naseem et al., 2011); (ii) siderophore biosynthesis, antibiotic production and sporulation in streptomycetes (Colson et al., 2008; Craig et al., 2012; Nazari et al., 2013; Rigali et al., 2006, 2008; Świątek et al., 2012a, b); (iii) altering the expression of virulence factors in pathogenic Escherichia coli (Barnhart et al., 2006; Sohanpal et al., 2004), amongst others (Konopka, 2012; Naseem et al., 2012).

The capability of actinomycetes to grow on chitin has long been studied (Jeuniaux, 1955) and has even become a traditional way to selectively isolate these micro-organisms from complex bacterial populations (Lingappa & Lockwood, 1961). Efficient and complete degradation of chitin requires the synergistic and coordinated action of enzymes with different substrate specificity. The chitinolytic and GlcNAc utilization systems are best documented in the model actinomycete Streptomyces coelicolor A3(2) (Colson et al., 2007, 2008; Kawase et al., 2006; Nothaft et al., 2003, 2010; Riediger et al., 2006; Saito et al., 2007, 2008, 2013; Schrepmpf, 1999; Świątek et al., 2012a; Zeltins & Schrepmpf, 1995). Strep. coelicolor possesses 11 chitinases, 9 belonging to family 18 and 2 belonging to family 19 of glycoside hyrolases (Saito et al., 2003). In addition, the genome of Strep. coelicolor encodes four chitin-binding proteins (CBPs), which are thought to facilitate chitinases accessibility by inducing structural changes upon binding to crystalline chitin (Itoh et al., 2002, 2003; Ohno et al., 1996; Schnellmann et al., 1994; Zeltins & Schrepmpf, 1995). Extracellular chitooligosaccharides and \( N,N' \)-diacetylchitobiose (GlcNAc) \(_2\) generated by chitinases would be further hydrolysed into GlcNAc by two predicted \( \beta \)-N-acetylglucosaminidases. In Strep. coelicolor, the uptake of GlcNAc \(_2\) (and with lesser efficiency chitooligosaccharides) is mediated by genes dasABC that encode an ATP-binding cassette (ABC) transporter. DasA is the high-affinity (GlcNAc) \(_2\)-binding component, and DasB and DasC form the ABC transporter permease (Colson et al., 2008; Saito et al., 2007). ATP hydrolysis for the transport of GlcNAc \(_2\) is performed by MsIK, the multiple sugar import ATPase (Saito et al., 2008). GlcNAc uptake is instead mediated via the phosphotransferase system (PTS), which consists of genes ptsL (enzyme I, EI), ptsH (HPr), crr (EIIA), nagF (NagF) and nagE2 (NagE2) (Nothaft et al., 2003, 2010). GlcNAc transported by the PTS (PTS\(_{GlcNAc}\)) is phosphorylated into \( N \)-acetylglucosamine-6-phosphate (GlcNAc-6P) by NagF once it enters the cell via the permease NagE2. Phosphorylation of GlcNAc originating from intracellular hydrolysis of GlcNAc \(_2\) by \( \beta \)-N-acetylglucosaminidases [DasD (Saito et al., 2013), SCO6032 and SCO2943] would be instead performed by the GlcNAc kinase NagK. GlcNAc-6P enters glycolysis by the successive deacetylation into glucosamine-6-phosphate (GlcN-6P) by the GlcNAc-6P deacetylase NagA, and subsequent deamination and isomerization of GlcN-6P into Fru-6P by NagB (Świątek et al., 2012a). The complete model of proteins and enzymes of Strep. coelicolor known or predicted to be involved in the catabolism of chitin and GlcNAc is presented in Fig. 1.

In Streptomyces coelicolor, the transcriptional control of genes belonging to the chitinolytic system and to GlcNAc metabolism involves the GntR-family regulatory protein DasR (Colson et al., 2007, 2008; Nazari et al., 2011, 2013; Rigali et al., 2004, 2006; Saito et al., 2007; Świątek et al., 2012a). DasR is necessary for the proper expression of genes involved in the degradation of the polymer chitin (Nazari et al., 2011, 2013), whereas it represses the expression of genes involved in chitooligosaccharide, (GlcNAc) \(_2\) and GlcNAc transport and catabolism (Colson et al., 2008; Rigali et al., 2006; Saito et al., 2007; Świątek et al., 2012a). Binding of GlcN-6P to the effector-binding domain of DasR impairs the DNA-binding ability of the repressor, which results in increased transcription of GlcNAc-utilization genes (Rigali et al., 2006).

The utilization of chitin and GlcNAc explains, at least in part, the evolutionary advantage of actinomycetes in soil colonization. While valuable research has been devoted to the understanding of chitin and GlcNAc metabolism in model Streptomyces species, it is unknown how applicable this knowledge is to distantly related actinomycetes. In this work we aimed to highlight the common and distinct features of chitin and GlcNAc utilization and their transcriptional control between the erythromycin producer Saccharopolyspora erythraea and Strep. coelicolor.

**METHODS**

**Bacterial strains, chemicals and culture conditions.** Bacterial strains and plasmids used in this study are listed in Table S1 (available in the online Supplementary Material). Saccharopolyspora erythraea NRRL23338 strain was grown on R2YE agar plates as described by Kieser et al. (2000) at 30 °C for sporulation. An agar piece about 1 cm\(^2\) was inoculated into a 250 ml flask containing 25 ml seed medium [50 g cornstarch l\(^{-1}\), 18 g soybean flour l\(^{-1}\), 13 g corn steep liquor l\(^{-1}\), 3 g NaCl l\(^{-1}\), 1 g (NH\(_4\))\(_2\)SO\(_4\) l\(^{-1}\), 1 g NH\(_4\)NO\(_3\) l\(^{-1}\), 5 g soybean oil l\(^{-1}\) and 6 g CaCO\(_3\) l\(^{-1}\), pH 6.8 to 7.0] and grown for 48 h at 34 °C and 200 r.p.m. Then 0.5 ml of the seed culture was added to a 500 ml flask containing 50 ml YEEM for the genomic DNA extraction (bacterial DNA kit, Qiagen), and incubation was continued at 34 °C and 200 r.p.m. for 4 days. E. coli strains were grown in LB medium at 37 °C. All media types were sterilized by autoclaving at 121 °C for 20 min. GlcNAc, GlcN-6P and chitin were purchased from Sigma-Aldrich. (GlcNAc) \(_2\) was purchased from V-LABORATORIES. Soluble chitin was prepared as described elsewhere (Murphy & Bleakley, 2012).

**Overexpression and purification of DasR-His\(_6\) protein.** SACE_0500/NCC_009142_0499 (dasR\(^{\text{rev}}\)) was amplified by PCR from Sacch. erythraea NRRL23338 genomic DNA using primers dasR-fw/dasR-sw (Table S2), and the 749 bp PCR product was then cloned into PET-28a (+) using restriction sites Ncol and HindIII, generating the recombinant plasmid pLCd001. After DNA sequencing, pLCd001 was transformed into E. coli BL21(DE3) for heterologous production of C-terminus His-tagged DasR (DasR-His\(_6\)). The E. coli cells were grown in 50 ml LB medium at 37 °C with 25 mg kanamycin ml\(^{-1}\) in an orbital shaker (250 r.p.m.) to an \( \text{OD}_{600} \) of 0.6. Expression was then induced with IPTG at a final concentration of 0.5 mM and followed by incubation at 30 °C for 6–8 h. For protein purification, cells were harvested by centrifugation and washed twice with PBS buffer (pH 8.0) and then broken by ultrasonic cell crusher. Cell debris and
membrane fractions were separated from the soluble fraction by centrifugation (45 min, 15 000 r.p.m., 4 °C). His-DasR was purified by Ni-NTA Superflow column (Qiagen). The protein was eluted with 250 mM imidazole (in 50 mM NaH2PO4, 300 mM NaCl, pH 8.0) (Fig. S1). The purified protein was dialysed in protein preservation buffer D (50 mM Tris, 0.5 mM EDTA, 50 mM NaCl, 20 % v/v glycerol, 1 mM DTT, pH 8.0) overnight at 4 °C and then stored at -280 °C before use. The quality of purified proteins was judged by SDS-PAGE. Protein concentration was determined with the Bradford reagent.

Electrophoretic mobility shift assay (EMSA). The upstream region (from -300 to +50 nt related to the start codon) of genes predicted to possess DasR responsive elements (dre) was amplified by PCR with gene-specific primers containing the common sequence 5'-AGCCAGTGGCGATAAG-3' (Table S2). To prepare biotin-labelled fragments, PCR products were used as templates for a second PCR using the universal primer of the common sequence whose 5'-end was labelled with biotin. The PCR products were identified by agarose gel electrophoresis and purified with a PCR purification kit (Shanghai Generay Biotech). The concentrations of biotin-labelled DNA probes were determined with a Biotek Synergy 2 multimode microplate reader at the wavelength of 260 nm (Bio-Tek Instruments). EMSAs were carried out according to the protocol accompanying the chemiluminescent EMSA kit (Beyotime Biotechnology). The binding reaction contained 10 mM Tris HCl pH 8.0, 25 mM MgCl2, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.01 % Nonidet P40, 50 μM poly[d(I-C)] ml⁻¹, 10 % glycerol (v/v). After DNA–protein incubation, samples were separated in a 6 % non-denaturing PAGE gel in ice-bathed 0.5 × Tris/borate-EDTA at 100 V and bands were detected by BeyoECL Plus. For short probes centred on dre, equimolar amounts of the two complementary single strand oligonucleotides were denatured at 94 °C for 5 min and allowed to anneal by lowering the temperature (1 °C min⁻¹) down to 25 °C in a standard PCR machine. In EMSAs, the DNA concentration was 10 nM, and the His-tagged DasR concentration was 1 μM. The reactions were performed at 20 °C for 15 min prior to loading in a non-denaturing PAGE gel.

Construction of the dasR in-frame deletion mutant. The gene mutation strategy was described elsewhere (Han et al., 2011). In order to construct an in-frame deletion of a 756 nt (GenBank accession no. NC_009142.1 553254–554009) fragment of the SACE_0500/NC_009142_0499 gene, two 1.5 kb DNA fragments of the adjacent region were amplified from Sacch. erythraea NRRL23338 genomic DNA by PCR, using the primer pairs dasR-up-fw/rev (the upstream fragment) and dasR-dw-fw/rev (the downstream fragment) (Table S2); then the PCR products were digested with EcoRI/BamHI and

**Fig. 1.** The chitinolytic system and GlcNAc metabolism pathways in *Strep. coelicolor* and *Sacch. erythraea*. The functions and references associated with proteins and enzymes shown here are described in Table 1. Proteins/enzymes in black are present in both *Strep. coelicolor* and *Sacch. erythraea*. Proteins/enzymes in grey and light grey are only present in *Strep. coelicolor* and in *Sacch. erythraea*, respectively. Chitinases with diagonal stripes are present in both strains, but the architecture of orthologous genes is not fully conserved. (GlcNAc)₂⁻₅, chitooligosaccharides; NH₃, ammonia; PEP, phosphoenolpyruvate.
Orthologous genes search. The search for transcription units (TUs) involved in chitin and GlcNAc utilization in *Sacch. erythraea* was facilitated by earlier investigations in *Strep. coelicolor*. The amino acid sequences of the GlcNAc/chitin-associated proteins of *Strep. coelicolor* were used as query sequences to identify their orthologues in acid sequences of the GlcNAc/chitin-associated proteins of *Strep. coelicolor* via the BLAST algorithm (Mount, 2007), the KEGG Sacch. erythraea were used as query sequences to identify their orthologues in *Strep. coelicolor* via the blast algorithm (Mount, 2007), the KEGG Sequence Similarity DataBase (SSDB) (Masoudi-Nejad et al., 2007), OrthoDB (Waterhouse et al., 2013) and OrtholugeDB (Whiteside et al., 2013).

Computational prediction of the DasR regulon in *Sacch. erythraea*. Significant research has been performed to functionally analyse DasR and to assess the full span of its regulon in *Strep. coelicolor* (Colson et al., 2007, 2008; Craig et al., 2012; Nazari et al., 2011, 2013; Rigel et al., 2004, 2006, 2008; Swiętek et al., 2012a) and in *B. subtilis* (Bertram et al., 2011; Resch et al., 2009, 2010). The fact that DasR possesses in its helix-turn-helix motif the key residues for proper recognition of *dre*-like sequences (Fig. 4) allows us to use data collected from studies in *Strep. coelicolor* to predict genes whose expression is likely to be controlled by this regulator in *Sacch. erythraea*, as recently performed for the nitrogen utilization regulator GlnR (Yao et al., 2014). PREDetector software (Hiard et al., 2007) was used to generate a position weight matrix with the *dre* upstream of genes that have been shown to be controlled by DasR via both in vitro (*dre/DasR interaction*) and in vivo (DasR-controlled expression) approaches. PREDetector was also used to scan the entire chromosome of *Sacch. erythraea* NRRL23338, and to retrieve reliable *dre*-like sequences upstream of genes involved in GlcNAc and chitin utilization.

**In silico analysis of genes involved in chitin and GlcNAc metabolism in *Sacch. erythraea***

Genes of *Sacch. erythraea* and *Strep. coelicolor* known or predicted to be involved in chitin degradation, and chitooligosaccharide and GlcNAc metabolism are listed in Table 1 and presented in Fig. 1. The most striking difference from *Strep. coelicolor* is the highly reduced number of genes that belong to the chitinolytic system in *Sacch. erythraea*. Only one gene (SACE_0100/NC_009142_0097) encodes a CBP compared to four CBPs in *Strep. coelicolor*; seven chitinase-encoding genes are present in *Sacch. erythraea* whereas *Strep. coelicolor* has 11 chitinases. Amongst the chitinases of *Sacch. erythraea*, two belong to the glycosyl hydrolases (GH) of family 18-A, two to family 18-B and three to family 18-C. The two predicted secreted β-N-acetylglucosaminidases of *Strep. coelicolor* are also conserved in *Sacch. erythraea* (SACE_3851 and SACE_0363). Importantly, *Sacch. erythraea* does not possess either the chitinases-encoding genes of GH family 19, which are the most active chitinases against soluble chitin (Kawase et al., 2006), or the genes required for chitooligosaccharide and (GlcNAc)₂ uptake encoded in *Strep. coelicolor* by the DasABC MsiK-dependent ABC-transporter. Genes *ngcEGF* encoding the putative alternative transporter of (GlcNAc)₂ (Xiao et al., 2002) are also absent in *Sacch. erythraea*. Finally, none of the four known or predicted intracellular β-N-acetylglucosaminidases of *Strep. coelicolor* (dasD, SCO0534, SCO2943 and SCO6032) is conserved in *Sacch. erythraea*, suggesting that *Sacch. erythraea* is not equipped for uptake and catabolism of dimer and oligomeric forms of GlcNAc but biased for the utilization of the monomer.

As opposed to *Strep. coelicolor*, when *Sacch. erythraea* is grown on minimal media containing colloidal chitin as the sole carbon source, biomass accumulation is extremely weak (Fig. 2a). Overall, this suggests that the massive loss of genes associated with chitin hydrolysis and chitooligosaccharide uptake and hydrolysis has a great impact on *Sacch. erythraea*’s ability to grow on chitin. The very low growth rate of *Sacch. erythraea* on MM chitin correlates with the absence of a clear halo of chitin hydrolysis even after prolonged (25 days) incubation (Fig. 2b). Similarly, no growth was observed when *Sacch. erythraea* was streaked on 7.5 mM Cell pellets were collected for RNA extraction as described elsewhere (Marcellin et al., 2013) at 45 min after the addition of GlcNAc.

RNA sequencing was performed as described earlier (Marcellin et al., 2013), with some modifications. Total RNA was extracted using two cycles of cellular lysis in RNase-free zirconia beads, followed by column purification using Qiagen RNAeasy. RNA quality was evaluated using a BioAnalyser (Agilent) and a Nanodrop 1000 (Thermo Scientific) prior to analysis. rRNA was removed with a Ribo-zero meta-bacterial mRNA enrichment kit (Eppicentre). Sequencing was performed on the RNA; sequencing analysis and read alignment were carried out as described elsewhere (Marcellin et al., 2013).

**RESULTS**

**In silico analysis of genes involved in chitin and GlcNAc metabolism in *Sacch. erythraea***

**Transcriptomic analysis.** *Sacch. erythraea* (NRRL2338) was grown in 2 l bioreactors (Applikon) in mineral medium MM-101 without Casamino acids, as described elsewhere (Marcellin et al., 2013). Approximately 0.5 ml of glycerol stock was used to inoculate a 500 ml baffled flask with 100 ml ISP 2 media incubated at 30 °C in a rotary shaker (INFORS HT) at 220 r.p.m. for 30 h. When the seed culture reached an OD₅₆₀ of 2.5 (early stationary phase), a second seed culture (1 l baffled flasks with 150 ml ISP 2) was inoculated to an initial OD₅₆₀ of 0.3 and incubated under the same culture conditions for 72 h. Cells were then centrifuged at 10000 r.p.m. at room temperature (Allegra X-15R; Beckman Coulter), washed and resuspended in MM-101 prior to inoculation. GlcNAc was added to the culture during mid-exponential phase to a final concentration of 7.5 mM. Cell pellets were collected for RNA extraction as described elsewhere (Marcellin et al., 2013) at 45 min after the addition of GlcNAc.
Table 1. Genes of GlcNAc and chitin utilization in \textit{Sacch. erythraea} and \textit{Strep. coelicolor}

For simplicity, the current gene annotation is not included. I, Amino acids identity; NA, not applicable; Qc, query coverage; S, amino acids similarity; !, indicates that the length of SCO and SACE sequences is different enough to affect the 'architecture' of the gene/protein and that one or more functional domains might therefore be absent.

<table>
<thead>
<tr>
<th>Gene SACE</th>
<th>Gene SACE</th>
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<th>Function</th>
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on MM with \((\text{GlcNAc})_2\) as the sole nutrient source (Fig. S2), which correlates with the lack of orthologues of *Strep. coelicolor* genes that encode the DasABC transporter and intracellular \(\beta\)-N-acetylglucosaminidases.

Instead, the genome of *Sacch. erythraea* contains the genetic information to utilize GlcNAc. *Sacch. erythraea* possesses the five PTS genes for GlcNAc transport and phosphorylation (\(\text{nagE2, nagF, crr, ptsH and ptsI}\)), and \(\text{nagA}\) required for the subsequent deacetylation of GlcNAc-6P into GlcN-6P. Remarkably, *Sacch. erythraea* does not have a classical \(\text{nagB}\) orthologue for deamination and isomerization of GlcN-6P into Fru-6P. The gene locus involved in linking aminosugar uptake and glycolysis is most likely SACE\_0498/NC\_009142\_0498. This gene was erroneously annotated as a \(\text{glmS}\)-like gene in the original annotation, encoding the enzyme that catalyses the opposite reaction of NagB that is the conversion of Fru-6P into GlcN-6P. In the recent reannotation of the *Sacch. erythraea* genome, the gene was reannotated as a phosphosugar isomerase (Marcellin et al., 2013). In reality, SACE\_0498/NC\_009142\_0498 is a hybrid \(\text{nagB/ glmS}\) ORF similar to the \(\text{nagB-II}\) genes present in Gram-positive bacteria (Yang et al., 2006). These NagB-II homologues contain the C-terminal domain of GlmS, but are only able to display the NagB catalytic activity and not the GlmS activity. The real \(\text{glmS}\) is instead SACE\_6768/NC\_009142\_6646. Except for \(\text{nagA}\), which is part of a bicistronic unit with the GlcNAc kinase (\(\text{nagK}\)), the organization of genes involved in GlcNAc metabolism is very different from *Strep. coelicolor* (Świątek et al., 2012; Rigali et al., 2004) (Fig. 3). \(\text{nagE2 (PTS E1C), nagF (PTS E1B), crr (PTS E1A) and ptsH (HPr)}\) are found within two divergently oriented TUs, whereas \(\text{ptsI (PTS E1)}\) is located elsewhere in the chromosome (Fig. 3). To demonstrate the functionality of all genes involved in GlcNAc metabolism, *Sacch. erythraea* was grown on GlcNAc-based minimal media (Figs 2a and S2). When chitin is combined with GlcNAc, the growth of *Sacch. erythraea* is similar to the growth with GlcNAc as the sole carbon source, with only a very weak loss (~10 %) of biomass accumulation (Fig. 2a).

### In silico analysis of DasR of *Sacch. erythraea*

In order to investigate the mechanism of the transcriptional control of GlcNAc and chitin utilization genes, the protein sequence of the *Strep. coelicolor* GlcNAc and chitin utilization regulator DasR (SCO5231, DasR\(_\text{SCO}\)) was used as a query sequence to identify its orthologue in *Sacch. erythraea*. SACE\_0500/NC\_009142\_0499 was identified as the best hit, with 62 and 72 % amino acid identity and similarity, respectively, and 97 % coverage. SACE\_0500/NC\_009142\_0499 is also identified as the orthologue of DasR\(_\text{SCO}\) in the KEGG Sequence Similarity DataBase (SSDB) (Masoudi-Nejad et al., 2007), OrthoDB (Waterhouse et al., 2013) and OrthologUEDB (Whiteside et al., 2013). Furthermore, a phylogenetic analysis with the full-length sequence of DasR\(_\text{SCO}\) and the six GntR/HutC regulators of *Sacch. erythraea* showed that SACE\_0500/NC\_009142\_0499
clusters with DasR\textsuperscript{Sco}, suggesting that both regulators had emerged from a common ancestor that is different from ancestors of other GntR/HutC members (Fig. 4a). When comparing the helix–turn–helix motif, the structural element mostly involved in DNA binding, we found that all seven residues anticipated by Resch et al. (2010) to directly interact with DasR responsive elements (dre) are conserved amongst SACE\textsubscript{0500}/NC\textsubscript{009142}_0499 and DasR\textsubscript{Sco} (Fig. 4b). This suggests that SACE\textsubscript{0500}/NC\textsubscript{009142}_0499 is certainly able to specifically recognize dre-like sequences and, importantly, that the sequences known to be bound by DasR\textsubscript{Sco} could be used to predict the DasR regulon in Sacch. erythraea (see below). Taken together, all these in silico data show that SACE\textsubscript{0500}/NC\textsubscript{009142}_0499 is unquestionably the closest homologue of the GlcNAc utilization repressor DasR studied in Streptomyces spp. and will be named DasR\textsubscript{Sery} throughout the rest of the paper. Interestingly, the first ORF of the predicted tri-cistronic operon that comprises nagE2 and ptsH encodes another GntR/HutC-subfamily regulator, which displays a helix–turn–helix motif very similar to dasR and will be called dasR2 (see Discussion).

**Identification of DasR responsive elements upstream of GlcNAc and chitin utilization genes**

The computational prediction of DasR-binding sites along the chromosome of Sacch. erythraea identified a series of dre-like sequences upstream of almost all TUs known or predicted to be involved in chitin and GlcNAc metabolism in Sacch. erythraea. The only two chi genes that do not possess a putative dre within their upstream region are SACE\textsubscript{0363}/NC\textsubscript{009142}_0363 (putative secreted β-N-acetylglucosaminidase) and SACE\textsubscript{5287}/ NC\textsubscript{009142}_5219 (putative chinotase family GH18-C) (Table 1).

The upstream regions of these TUs (350 bp fragments) were incubated with the pure His-tagged DasR\textsubscript{Sery} (Fig. S1) in order to detect possible DNA/protein interactions via gel retardation assays. EMSAs revealed that His-tagged DasR\textsubscript{Sery} was able to interact with all upstream regions tested (Fig. 5a). Mobility retardation was also observed with a 39 bp DNA fragment centred on the predicted cis-acting element of ptsI and chiA suggesting that the computationally predicted 16 bp dre-like sequence is necessary and sufficient for DasR\textsubscript{Sery} binding as deduced from Strep. coelicolor (Rigali et al., 2008; Colson et al., 2007) and Bacillus subtilis studies (Bertram et al., 2011; Resch et al., 2009) (Fig. 5a). The compilation of all dre identified in Sacch. erythraea highlighted the consensus nntTGGTCTAGACCA\textsubscript{n}, which is highly similar to the anTGGTCTAGACC\textsubscript{Ant} consensus deduced from Strep. coelicolor studies (Fig. 5b).

GlcN-6P was reported to inhibit the DNA-binding capability of DasR in Strep. coelicolor (Rigali et al., 2008) and of its orthologue NagR in B. subtilis (Bertram et al. 2011). In
Sacch. erythraea, EMSA analyses revealed that GlcN-6P was able to modify the DNA-binding of DasR\textsuperscript{Ser\textsubscript{70}} to dre\textsuperscript{ptsI} (Fig. 5c). However, the GlcN-6P addition to the in vitro reaction did not result in the loss of the shifted band that corresponds to the DasR\textsuperscript{Ser\textsubscript{70}}/dre\textsuperscript{ptsI} complex, but caused the occurrence of additional shifted bands. The multiple bands suggest that DasR\textsuperscript{Ser\textsubscript{70}} would not behave in a classical operator/repressor mode with the loss of the DNA-binding ability upon ligand binding, but would undergo an allosteric modification that would result in a different mode of binding to DNA like that suggested by Resch et al. (2010) for NagR in B. subtilis (see Discussion).

**Expression of GlcNAc and chitin utilization genes is repressed by DasR and induced by GlcNAc**

To study the regulatory role of DasR on the expression of identified target genes, real-time RT-PCR was performed using RNAs extracted at two time points (12 and 36 h) from the parental strain Sacch. erythraea NRRL 23338 and the dasR mutant strain grown in balanced minimal Evans medium (Fink et al., 2002). As shown in Fig. 6, the deletion of dasR led to higher expression levels of all GlcNAc-assimilation-related genes. Indeed, transcription of nagK, nagB-II, nagA, pstI, nagF, crr, dasR2, nagE2 and ptsH after 12 h of growth was quantified to about 100-, 50-, 57-, 23-, 4-, 3-, 2.5-, 2.4- and 2-fold higher in the dasR null mutant compared to the parental strain, respectively. The exception was observed for glmS (SACE\textsubscript{6768}/NC\textsubscript{009142}_6646), which only displays a slight enhanced expression at both time points. The peculiar expression pattern of glmS suggests additional transcription control levels aside from the one exerted by DasR (see Discussion). The increased expression of genes involved in the uptake of GlcNAc (PTS\textsubscript{GlcNAc}) and GlcNAc catabolism (nagKA and nagB-II) in the dasR mutant correlates with the increased biomass accumulation of strain Sacch. erythraea ΔdasR compared to the wild-type strain when grown on MM media with GlcNAc or GlcNAc+chitin as carbon and nitrogen source (Fig. 2a). Similarly, real-time RT-PCR revealed strong enhanced expression of most genes involved in chitin degradation in the dasR null mutant compared to the parental strain (Fig. 6). chiH, chiB, chiA, hexA, chiD and chiE showed a maximum of 28-, 15-, 10-, 5-, 3.6- and 4-fold increased expression, respectively, as a consequence of dasR deletion. SACE\textsubscript{3887}/NC\textsubscript{009142}_3884 showed only enhanced expression in RNA samples from the dasR mutant collected at 36 h. Surprisingly, chiB, which possesses two dre-like sequences in its upstream region (Fig. 3), did not show significant expression changes.
motifs of SACE_0500/NC_009142_0499 (DasR) from Saccharopolyspora erythraea in dre (1922). Microbiology. nagK genes, as well as dasR from Strep. coelicolor defined medium increased transcription of all PTS GlcNAc that supply of 7.5 mM GlcNAc to glucose chemically. Additionally, genome-wide transcriptional analysis revealed (i) one chitin-binding protein, (ii) two GH18-A chitinases, (iii) two GH18-B chitinases, (iv) three GH18-C chitinases and (v) two predicted secreted β-N-acetylgalactosaminidases. The inability of Sacch. erythraea to utilize chitin despite a complete chitinolytic arsenal is intriguing. We do not think that it could be attributed to low expression levels of chitinolytic genes, as even with constitutive expression of chi genes, the Sacch. erythraea ΔdasR mutant strains do not display sufficient chitinase activity to visualize haloes around colonies in MM supplied with soluble chitin (Fig. 2b). Even in the presence of GlcNAc, which activates expression of the chitinolytic system, we could not visualize haloes of chitin degradation by either Sacch. erythraea wild-type or its dasR null mutant (Fig. S3b), suggesting a very low global chitinase activity.

DISCUSSION

Swiontek Brzezinska and colleagues demonstrated that a mean of 80 and 85% of actinomycetes in sandy soil and in farm-land soil, respectively, display chitin hydrolysis (Brzezinska et al., 2009). Chitinolytic actinomycetes also made up a large fraction of total actinomycetes in water samples (mean of 73%), whereas in silt and sandy sediments the percentages of chitinolytic actinomycetes drop to 23 and 15%, respectively (Brzezinska et al., 2009). We showed here that Sacch. erythraea cannot properly grow in MM with chitin and (GlcNAc)2 as sole carbon and nitrogen source, despite having all the genetic elements required for chitin and chitooligosaccharide hydrolysis, i.e. (i) one chitin-binding protein, (ii) two GH18-A chitinases, (iii) two GH18-B chitinases, (iv) three GH18-C chitinases and (v) two predicted secreted β-N-acetylgalactosaminidases. The inability of Sacch. erythraea to utilize chitin despite a complete chitinolytic arsenal is intriguing. We do not think that it could be attributed to low expression levels of chitinolytic genes, as even with constitutive expression of chi genes, the Sacch. erythraea ΔdasR mutant strains do not display sufficient chitinase activity to visualize haloes around colonies in MM supplied with soluble chitin (Fig. 2b). Even in the presence of GlcNAc, which activates expression of the chitinolytic system, we could not visualize haloes of chitin degradation by either Sacch. erythraea wild-type or its dasR null mutant (Fig. S3b), suggesting a very low global chitinase activity.

The most likely reason for the inability to utilize chitin and (GlcNAc)2 could be the loss of the DasABC transporter for (GlcNAc)2 and intracellular β-N-acetylgalactosaminidases, which would impair feeding on chitooligosaccharides. This is further supported by the fact that other pseudonocardiaeae, such as Saccharothrix espanaensis, Actinosynnema mirum, Amycolatopsis species, Kutzeria species and, more importantly, the closely related strain Saccharopolyspora spinosa, have conserved in their genome the orthologues of dasA, dasB and dasC required for (GlcNAc)2 transport. Fair growth of Sacch. spinosa was reported when inoculated on chitin-agar plates (Mertz & Yao, 1990). However, the inactivation of dasA in Strep. coelicolor, either via gene interruption (Colson et al., 2008) or via gene deletion (Saito et al., 2007), strongly enhances the chitinolytic activity. A plausible explanation for the deficiency of a chitinolytic activity in Sacch. erythraea could also be attributed to the absence of GH19 family chitinases, which are the most efficient enzymes for hydrolysis of soluble chitin (Kawase et al., 2006). However, many actinomycetes

in the dasR mutant after 12 h of growth, although a 3.5-fold increased expression was quantified at 36 h (Fig. 6).

Additionally, genome-wide transcriptional analysis revealed that supply of 7.5 mM GlcNAc to glucose chemically defined medium increased transcription of all PTS GlcNAc genes, as well as nagK, nagA and nagB-II, after 45 min of GlcNAc addition (Fig. 7). While chb1 and dasR2 displayed the maximal and minimal positive response, respectively, only glmS was downregulated upon GlcNAc addition (Fig. 7). The exception observed for glmS was anticipated as it encodes an enzyme of aminosugar anabolism and not catabolism; therefore, logically it would be downregulated by the aminosugar supply. Surprisingly, all genes that belong to the chitinolytic system showed enhanced expression upon GlcNAc supply except for the two genes (SACE_0363/NC_009142_0363 and SACE_6557/NC_009142_6433) that do not possess a dre site within their upstream region (Fig. 7). Thus, in contrast to the situation observed in Strep. coelicolor, GlcNAc does not repress but instead induces the expression of genes involved in the degradation of chitin, the polymer of GlcNAc. However, the increased expression of chitinase-encoding genes in the dasR mutant (Fig. 6), even combined with GlcNAc supply (Fig. S3b), is not sufficient for visualization of a halo of chitinolytic activity in MM chitin (Fig. 2b) or to allow normal growth of Sacch. erythraea when chitin is the only nutrient source available (Fig. 2a).

**Fig. 4. In silico analysis of Sacch. erythraea DasR.** (a) Phylogeny analysis of GntR/HutC subfamily regulators of Sacch. erythraea and DasR from Strep. coelicolor. Note that SACE_0500/NC_009142_0499 (DasR^Sey) and SCO5231 (DasR^Scn) emerge from a common ancestor that is different from other GntR/Hutc members of Sacch. erythraea. (b) Alignment of helix–turn–helix motifs of SACE_0500/NC_009142_0499 (DasR^Sey) and SCO5231 (DasR^Scn). The seven residues predicted to be involved in dre-binding (Resch et al., 2010) are conserved between Sacch. erythraea and from Strep. coelicolor.
and other bacteria do not possess GH19 family chitinases but are still able to grow on media with chitin as the sole nutrient source. Finally, it should be noted that none of the chitinolytic genes of *Sacch. erythraea* has been investigated in biochemical studies. It is therefore possible that genes encoding chitinases in *Sacch. erythraea* have undergone a series of deletions/mutations that modify their chitinolytic properties. This hypothesis is for instance supported by the comparison of the amino acid sequence of *Sacch. erythraea* and *Strep. coelicolor* ChiA and ChiE-like proteins. Indeed, ChiA and ChiE of *Sacch. erythraea* (SACE_5394/NC_009142_5326 and SACE_6557/NC_009142_6433) both lack the type II carbohydrate-binding domain and the fibronectin type 3 domain that are both present in *Strep. coelicolor* (SCO5003 and SCO5954). Similarly, ChiB of *Sacch. erythraea* (SACE_2232/NC_009142_2202) lacks the type IV carbohydrate-binding domain present in SCO5673 of *Strep. coelicolor*. However, the lack of the carbohydrate-binding domain does not affect the ability of GH18 family enzymes to degrade soluble chitin (Tjoelker *et al.*, 2000).

In addition, the catalytic cleft containing catalytic residues DXDXE (Synstad *et al.*, 2004) is conserved in six out of the seven predicted GH18 family chitinases of *Sacch. erythraea* (Fig. S4).

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**Fig. 5.** DNA- and ligand-binding properties of *Sacch. erythraea* DasR. (a) EMSAs with pure His-tagged DasR<sub>Sery</sub> and dre probes. Probes are either 350 bp fragments corresponding to the upstream region (UR, -300 to +50 nt upstream and downstream of the translational start) of genes predicted to be controlled by DasR<sub>Sery</sub> or 39 bp annealed oligonucleotides centred on the predicted 16 bp dre. The promoter region of crp (SACE_0329/NC_009142_0329) was used for the control (ctrl). (b) Weblogo representation of *Strep. coelicolor* and *Sacch. erythraea* consensus dre sites. (c) Identification of GlcN-6P as allosteric effector of DasR<sub>Sery</sub>. EMSAs were performed with dre<sup>ptsI</sup> as the probe. + and – indicate the presence or absence of 1 μM His-tagged DasR<sub>Sery</sub>. GlcNAc or GlcN-6P was added to the dre<sup>ptsI</sup>–DasR binding reaction at a final concentration of 50, 100 and 200 mM. Arrow 1 indicates free DNA probe. Addition of GlcN-6P induced different retardation signals (lanes 3, 4 and 5, indicated by arrows 2, 3 and 4) suggesting that the DasR/GlcN-6P complex does not fully dissociate from dre<sup>ptsI</sup>.
Interestingly, DasABC transporter would contribute more significantly. Chitin is probably the result of a combination of the ‘pre-conditioned’ by a culture of when spores were spotted onto an MM chitin previously. The reason why Sacch. erythraea is an abundant nutrient source, Sacch. erythraea could be considered as a ‘cheater’ organism waiting for the release of GlcNAc by chitin degradation ‘helpers’.

Since the discovery of cryptic gene clusters in streptomycetes and other actinomycetes, microbial screening programmes have started to become newly fashionable pursuits. Because the capability of actinomycetes to feed on chitin as the sole nutrient is thought to be so commonplace, powdered chitin-agar-based protocols were and still are largely employed for promoting growth of rare actinomycetes present in the soil samples and simultaneously suppressing/hindering the contaminant bacterial/fungal colonies (Barke et al., 2010; Bredholt et al., 2008; Hsu & Lockwood, 1975; Khanna et al., 2011; Lingappa & Lockwood, 1961). From the industrial point of view, the erythromycin-producer Sacch. erythraea is a very important actinomycete, and orthologous gene search and syntenic investigations have revealed that the combined absence of GH19 chitinases, intracellular β-N-acetylglucosaminidases and the DasABC transporter is also a common feature of other rare actinomycetes, such as Saccharomonospora and several Pseudonocardia species. The inability of actinomycetes to grow on chitin-containing media might be more widespread than expected. Actinomycetes isolation protocols only based on chitin supplemented media might thus miss many promising biopharmaceutical compound producers.

In addition to the enzymic and transporter components of chitin and GlcNAc utilization, the transcriptional regulatory system in Sacch. erythraea also has its own particularities compared to the situation described in Strep. coelicolor. The fact that the large majority of genes that compose the chitinolytic system are upregulated in the dasR mutant is a first major difference from what had been described in Strep. coelicolor (Nazari et al., 2011, 2013). The opposing regulatory roles of DasRSen and DasRSso on chi gene transcription challenge the idea that in Strep. coelicolor DasR would control gene expression only via a classical operator/repressor mode. At least in cases of genes activated by DasR, their transcriptional control would involve

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**Fig. 6.** Effect of dasR deletion on the expression of genes involved in GlcNAc and chitin metabolism in Sacch. erythraea. RNAs were extracted from Sacch. erythraea NRRL2338 (black bars) and its dasR mutant strain (grey bars) grown for 12 and 36 h in balanced Evans medium. Relative transcript levels were normalized to the 16S rRNA at the corresponding time points. The relative value for the expression of each gene at 12 h in Sacch. erythraea NRRL2338 was arbitrarily assigned as value 1. Data are shown as the mean ± SD from three independent experiments, each with triplicate samples, using distinct cDNA preparations for each RNA sample.
additional and still unknown interacting partners and/or DasR would behave differently according to the binding of different allosteric effectors. Interestingly, in terms of allosteric effector, GlcN-6P did not induce a similar effect on DasR DNA binding as observed in *Strep. coelicolor*, where DasR<sup>Sco</sup> does not bind to dre once complexed to its ligand (Rigali <sup>et al.</sup>, 2006). Instead, GlcN-6P induced several shifted bands in EMSAs, which could be due to allosteric modifications that would still conserve the DNA-binding capability of DasR<sup>Sery</sup>, but the DNA-binding domains would be reoriented and forced apart as described for the orthologue NagR in *B. subtillis* (Resch <sup>et al.</sup>, 2010).

Indeed, Resch and colleagues showed that GlcNAc-6P binding to NagR leads to a 120° rotation of the DNA-binding domains that is best described as a jumping-jack-like motion and this would allow each monomer of the regulator to bind to two different dres (Resch <sup>et al.</sup>, 2010). Studies on the allosteric effectors of DasR and NagR in *Strep. coelicolor* and *B. subtillis* give contradicting results based on the methodology used for EMSAs (Gaugué <sup>et al.</sup>, 2014; S. Rigali, unpublished data). Extensive work is still needed to properly identify all allosteric effectors of DasR/NagR orthologues and how each of them modulates the DNA-binding property of the regulator.

A second major difference with *Strep. coelicolor* is that the addition of GlcNAc activates *Sacch. erythraea* chi gene expression, inferring that the presence of the monomer GlcNAc stimulates the catabolism of the polymer chitin. The absence of orthologues of chitooligosaccharide transporters in *Sacch. erythraea*, and the unique possibility of importing chitin by-products via the PTS<sub>GlcNAc</sub>, would provide a good explanation as to why GlcNAc activates the expression of chi genes in this organism. In addition, one gene that positively responds to GlcNAc, and belongs to the DasR regulon, deserves further attention. The first ORF of the TU of nagE2 and ptsH encodes another regulator of the GntR/HutC family (Hoskisson & Rigali, 2009; Rigali <sup>et al.</sup>, 2002), temporarily named here DasR2. The overall amino acid conservation between DasR<sup>Sery</sup> and DasR2 is rather low, with 40 and 56% of identity and similarity, respectively. However, the DNA-binding domains are more conserved than the ligand-binding domains between the two GntR/

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**Fig. 7.** Effect of GlcNAc supply on the expression of genes involved in GlcNAc and chitin metabolism in *Sacch. erythraea*. The values represent the transcriptional pattern of the GlcNAc operon and genes involved in GlcNAc metabolism. RNA was extracted 45 min after GlcNAc induction in glucose-based minimal medium MM101. Transcription values were log<sub>2</sub> transformed. Data shown are means of two independent experiments.
HutC family regulators, and six out of the seven residues predicted to be involved in DNA binding are conserved between SACE_0500 (DasR) and SACE_2060 (DasR2). DasR<sup>Sery</sup> thus represses the expression of a transcription factor that could compete for the same or similar cis-acting elements but respond to a different allosteric effector. The search of DNA motifs bound by DasR2 and the ligand affecting its DNA-binding ability is under investigation.

Finally, the inactivation of dasR is known to affect secondary metabolite production and sporulation in <i>Streptomyces</i> (Rigali <i>et al.</i>, 2006, 2008). Indeed, <i>das</i> stands for deficient in aerial hyphae and spore formation (Seo <i>et al.</i>, 2002), highlighting that the DasR regulatory protein has been discovered through developmental studies before uncovering its physiological function in the control of GlcNAc metabolism. Similarly, GlcNAc supply also triggers or inhibits sporulation and secondary metabolite production in <i>Streptomyces</i> depending on the culture conditions (Rigali <i>et al.</i>, 2008). The preliminary observation of the <i>Sacch. erythraea ΔdasR</i> mutant strain grown in GlcNAc-containing media also suggests impaired development compared to the wild-type strain (Fig. 53b). Accurate description of the development of the <i>Sacch. erythraea ΔdasR</i> mutant is currently under investigation, as well as the full characterization of the DasR regulon, in order to highlight how this global regulator mediates cross-talk between primary and secondary metabolism in <i>Sacch. erythraea</i>.

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