

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 (PTS^{GlcNAc}); 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.

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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-acetylglucosamine (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.

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Abbreviations: EMSA, electrophoretic mobility shift assay; Fru-6P, fructose-6-phosphate; GlcN-6P, glucosamine-6-phosphate; GlcNAc, *N*-acetylglucosamine; (GlcNAc)₂, *N,N'*-diacetylchitobiose; PTS, phosphotransferase system; RT-PCR, reverse transcriptase PCR; TU, transcription unit.

The transcriptomic data have been deposited at the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO), accession number GSE56541. Four supplementary figures and two supplementary tables are available with the online version of this paper.

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; Rigali *et al.*, 2006; Saito *et al.*, 2003, 2007, 2008, 2013; Schrempf, 1999; Świątek *et al.*, 2012a; Zeltins & Schrempf, 1995). *Strep. coelicolor* possess 11 chitinases, 9 belonging to family 18 and 2 belonging to family 19 of glycoside hydrolases (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 & Schrempf, 1995). Extracellular chitoooligosaccharides and *N,N'*-diacetylchitobiose [(GlcNAc)₂] generated by chitinases would be further hydrolysed into GlcNAc by two predicted secreted β -*N*-acetylglucosaminidases. In *Strep. coelicolor*, the uptake of (GlcNAc)₂ (and with lesser efficiency chitoooligosaccharides) 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 *ptsI* (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 β -*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 the 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 *Strep. 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 chitoooligosaccharide, (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*, 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 cm² 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 (NH₄)₂SO₄ l⁻¹, 1 g NH₄NO₃ l⁻¹, 5 g soybean oil l⁻¹ and 6 g CaCO₃ 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 YEME 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 (Murthy & Bleakley, 2012).

Overexpression and purification of DasR-His₆ protein. SACE_0500/NC_009142_0499 (*dasR*^{S_{err}}) was amplified by PCR from *Sacch. erythraea* NRRL23338 genomic DNA using primers *dasR*-fw/rev (Table S2), and the 749 bp PCR product was then cloned into pET-28a(+) using restriction sites *Nco*I and *Hind*III, generating the recombinant plasmid pLCd001. After DNA sequencing, pLCd001 was introduced into *E. coli* BL21(DE3) for heterologous production of C-terminus His-tagged DasR (DasR-His₆). 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 OD₆₀₀ of 0.6. Expression was then induced with IPTG at a final concentration of 0.5 mM and followed by incubation at 20 °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

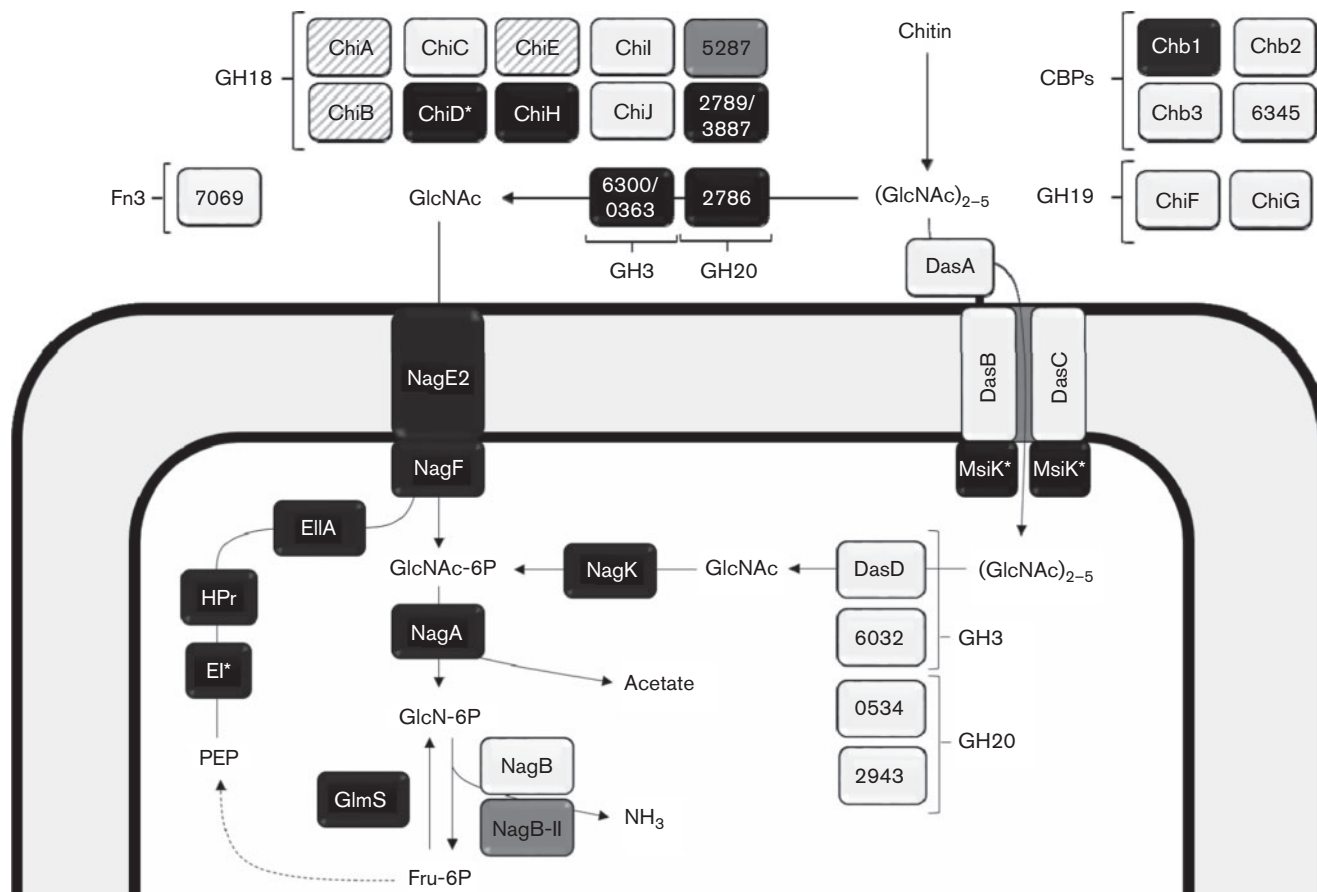


Fig. 1. The chitinolytic system and GlcNAc metabolism pathways in *Strep. coelicolor* and *Sacch. erythraea*. The functions and references associated with proteins and enzymes shown here are described in Table 1. Proteins/enzymes in black are present in both *Strep. coelicolor* and *Sacch. erythraea*. 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.

membrane fractions were separated from the soluble fraction by centrifugation (45 min, 15 000 r.p.m., 4 °C). His₆-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 -80 °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 Genaray 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 µg 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/Bam*HI and

KpnI/HindIII, respectively, and subsequently inserted into the corresponding sites of pUCTSR, creating the pUC-D0500 knockout plasmid. The resulting mutation plasmid was transferred into *Sacch. erythraea* NRRL23338 by PEG-mediated transformation. Preparation of protoplasts was described elsewhere (Han *et al.*, 2011). For the linearized fragment homologous recombination strategy, the SACE_0500/NC_009142_0499 gene in *Sacch. erythraea* was replaced with the thiostrepton resistance gene cassette. The selected mutants were verified by PCR and DNA sequencing.

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 *Sacch. erythraea* 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; Rigali *et al.*, 2004, 2006, 2008; Świątek *et al.*, 2012a) and in *B. subtilis* (Bertram *et al.*, 2011; Resch *et al.*, 2009, 2010). The fact that DasR^{Sery} 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^{Sco} 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.

Real-time reverse transcriptase PCR (RT-PCR). *Sacch. erythraea* NRRL 23338 and the *dasR* mutant strain was grown for 2 days at 30 °C in seed medium, and then 1% of the seed culture was inoculated into balanced minimal Evans medium (Fink *et al.*, 2002). RNA was extracted and purified as described elsewhere (Li *et al.*, 2013). About 1 µg total RNA was reverse transcribed using a PrimeScript RT reagent kit with gDNA Eraser (Takara). For real-time RT-PCR, a SYBR premix Ex *Taq* GC kit (Perfect Real-time; Takara) was used and about 100 ng cDNA was added in a 20 µl volume of PCR mixture. The PCR was conducted using a CFX96 real-time system (Bio-Rad) and the PCR conditions were 95 °C for 5 min; then 40 cycles of 95 °C for 10 s, 60 °C for 10 s and 72 °C for 30 s; finally followed an extension at 72 °C for 10 min.

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 OD₄₅₀ of 2.5 (early stationary phase), a second seed culture (1 l baffled flasks with 150 ml ISP 2) was inoculated to an initial OD₄₅₀ of 0.3 and incubated under the same culture conditions for 72 h. Cells were then centrifuged at 10 000 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.

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 RNeasy. 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*

Genes of *Sacch. erythraea* and *Strep. coelicolor* known or predicted to be involved in chitin degradation, and chito-oligosaccharide 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/NC009142_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 chito-oligosaccharide and (GlcNAc)₂ uptake encoded in *Strep. coelicolor* by the DasABC MsiK-dependent ABC-transporter. Genes *ngcEFG* 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 chito-oligosaccharide 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

Table 1. Genes of GlcNAc and chitin utilization in *Sacch. erythraea* and *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.

Gene	SACE	SCO	I/S (Qc)	Function	Selected references
PTS-mediated GlcNAc transport and phosphorylation					
<i>nagE2</i>	2059	2907	54/66 (95)	NagE2, PTS enzyme EIIC transmembrane component	Nothaft <i>et al.</i> (2003, 2010)
<i>nagF</i>	2060	2905	61/80 (85)	NagF, PTS enzyme EIIB component	
<i>crr</i>	2061	1390	57/71 (95)	EIIA, PTS enzyme EIIA component	
<i>ptsH</i>	2058	5841	51/68 (94)	HPr, PTS phosphocarrier protein	
<i>pstI</i>	7368	1391	52/63 (99)	EI, PTS enzyme EI component	
GlcNAc transport and metabolism					
<i>nagA</i>	7184	4284	57/67 (98)	NagA, GlcNAc-6P deacetylase	Świątek <i>et al.</i> (2012a, b)
<i>nagB</i>	–	5236	NA	NagB, GlcN-6P deaminase/isomerase	
<i>nagK</i>	7183	4285	50/64 (80)	NagK, GlcNAc kinase	–
<i>glmS</i>	6768	2789	44/61 (100)	GlmS, glucosamine-Fru-6P aminotransferase	Foley <i>et al.</i> (2008)
<i>glmS</i>	–	4740	68/77 (100)	GlmS, glucosamine-Fru-6P aminotransferase	–
<i>nagBII</i>	0498	–	NA	GlcN-6P deaminase/isomerase	–
Chito-oligosaccharide transport and intracellular catabolism					
<i>dasA</i>	–	5232	NA	DasA, ABC-transporter (GlcNAc) ₂₋₅ binding protein	Saito <i>et al.</i> (2007); Colson <i>et al.</i> (2008)
<i>dasB</i>	–	5233	NA	DasB, (GlcNAc) ₂₋₅ ABC-transporter permease	
<i>dasC</i>	–	5234	NA	DasC, (GlcNAc) ₂₋₅ ABC-transporter permease	
<i>dasD</i>	–	5235	NA	DasD, (GlcNAc) ₂₋₅ β -N-acetylglucosaminidase GH3	Saito <i>et al.</i> (2013)
–	–	6032	NA	β -N-acetylglucosaminidase GH3	–
–	–	2943	NA	β -N-acetylglucosaminidase GH20	–
–	–	0534	NA	β -N-acetylglucosaminidase GH20	–
<i>msiK</i>	0454	4240	63/76 (99)	Multiple sugar import ATPase	Saito <i>et al.</i> (2008)
Chitin binding/degradation					
<i>chiA</i>	5394	5003	57/73 (51!)	ChiA, chitinase family GH18-B	Kawase <i>et al.</i> (2006)
<i>chiB</i>	2232	5673	66/77 (55!)	ChiB, chitinase family GH18-B	Morimoto <i>et al.</i> (1997)
<i>chiC</i>	–	5376	NA	ChiC, chitinase family GH18-A	Kawase <i>et al.</i> (2006)
<i>chiD</i>	6558	1429	40/54 (89)	ChiD, chitinase family GH18-A	
<i>chiE</i>	6557	5954	40/54 (55!)	ChiE, chitinase family GH18-A	Huang <i>et al.</i> (2012)
<i>chiF</i>	–	7263	NA	ChiF, chitinase family GH19	Yang <i>et al.</i> (2005)
<i>chiG</i>	–	0482	NA	ChiG, chitinase family GH19	Heggset <i>et al.</i> (2009)
<i>chiH</i>	1076	6012	34/49 (67)	ChiH, chitinase family GH18-C	Nazari <i>et al.</i> (2011)
<i>chiI</i>	–	1444	NA	ChiI, chitinase family GH18-A	Tsujibo <i>et al.</i> (2002)
<i>chiJ</i>	–	2503	NA	ChiJ, chitinase family GH18-B	Sampei <i>et al.</i> (2004)
–	3887	2799	38/51 (88)	Putative chitinase family GH18-C	Karlsson & Stenlid (2009)
–	5287	–	36/45 (88)		
–	–	7069	NA	Fibronectin type 3 domain chitinase FnIII	Watanabe <i>et al.</i> (1990)
<i>hexA</i>	3851	2786	60/72 (98)	Secreted β -N-acetylglucosaminidase GH20	Mark <i>et al.</i> (1998)
<i>bglX</i>	0363	6300	50/64 (96)	Secreted β -N-acetylglucosaminidase GH3	Tsujibo <i>et al.</i> (1998)

Table 1. cont.

Gene	SACE	SCO	I/S (Qc)	Function	Selected references
<i>chb</i>	0110	2833	51/63 (99)	Chitin-binding protein	Schrempf (1999); Schnellmann <i>et al.</i> (1994); Itoh <i>et al.</i> (2002); Ohno <i>et al.</i> (1996)
<i>chb2</i>	–	7225	NA	Chitin-binding protein	
<i>chb3</i>	–	0481	NA	Chitin-binding protein	
–	–	6345	NA	Chitin-binding protein	
Regulation					
<i>dasR</i>	0500	5231	64/72 (97)	GlcNAc utilization regulator	Rigali <i>et al.</i> (2004, 2006)
<i>dasR2</i>	2060	–	NA	GntR-family transcriptional regulator	–

on MM with (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 β -N-acetylglucosaminidases.

Instead, the genome of *Sacch. erythraea* contains the genetic information to utilize GlcNAc. *Sacch. erythraea* possess the five PTS genes for GlcNAc transport and phosphorylation (*nagE2*, *nagF*, *crr*, *ptsH* and *ptsI*), and *nagA* required for the subsequent deacetylation of GlcNAc-6P into GlcN-6P. Remarkably, *Sacch. erythraea* does not have a classical *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 *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 *nagB/glmS* ORF similar to the *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 *glmS* is instead SACE_6768/ NC_009142_6646. Except for *nagA*, which is part of a bicistronic unit with the GlcNAc kinase (*nagK*), the organization of genes involved in GlcNAc metabolism is very different from *Strep. coelicolor* (Świątek *et al.*, 2012a; Rigali *et al.*, 2004) (Fig. 3). *nagE2* (PTS EIIC), *nagF* (PTS EIIB), *crr* (PTS EIIA) and *ptsH* (HPr) are found within two divergently oriented TUs, whereas *ptsI* (PTS EI) 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^{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^{SCO} in the KEGG Sequence Similarity DataBase (SSDB) (Masoudi-Nejad *et al.*, 2007), OrthoDB (Waterhouse *et al.*, 2013) and OrtholugeDB (Whiteside *et al.*, 2013). Furthermore, a phylogenetic analysis with the full-length sequence of DasR^{SCO} and the six GntR/HutC regulators of *Sacch. erythraea* showed that SACE_0500/NC_009142_0499

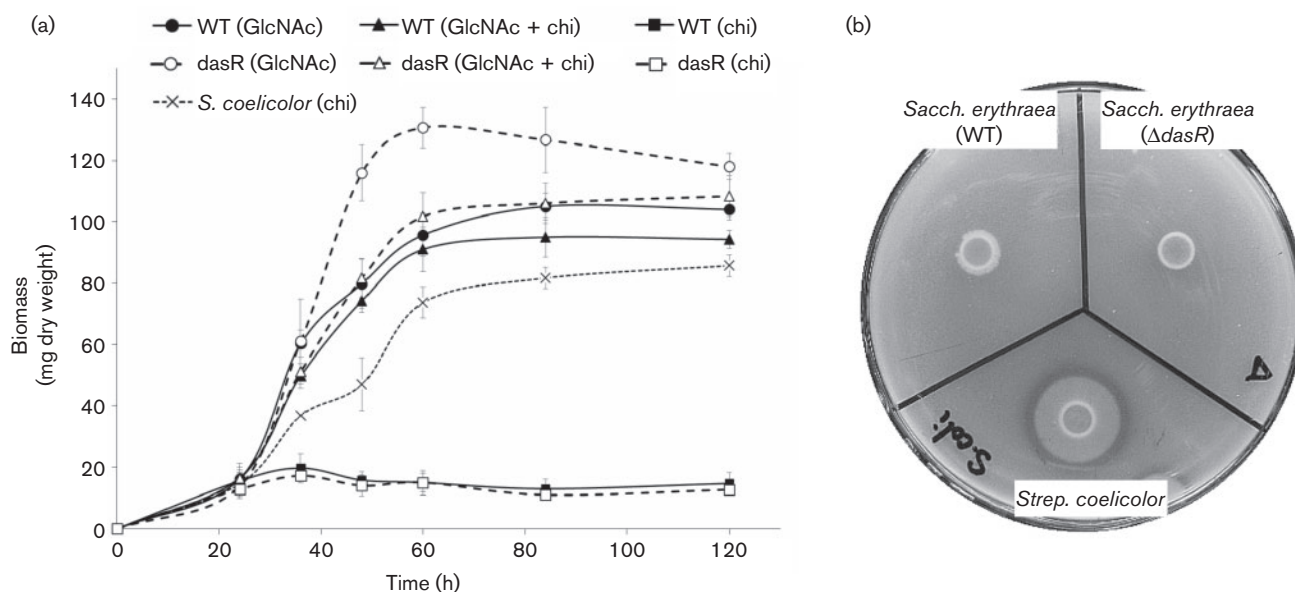


Fig. 2. Growth and viability of *Sacch. erythraea* with GlcNAc and chitin as sole nutrient sources. (a) *Sacch. erythraea* wild-type and Δ *dasR* mutant strains, as well as *Strep. coelicolor* M145, were streaked onto MM agar plates (covered with cellophane discs) supplemented with 2% (w/v) GlcNAc or chitin (chi) as sole carbon sources, or 1% GlcNAc and 1% chitin when both nutrient sources were added together. The biomass of mycelia was harvested from the cellophane at seven time points as indicated. (b) Spores (OD_{450} 1.0, 50 μ l) of *Sacch. erythraea* wild-type and Δ *dasR* mutant strains, as well as *Strep. coelicolor* M145, were spotted on MM agar supplied with 2% (w/v) chitin as the sole carbon source. Note that a clear halo of chitin hydrolysis is only visible for *Strep. coelicolor*.

clusters with DasR^{Sc}, 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_0500/NC_009142_0499 and DasR^{Sc} (Fig. 4b). This suggests that SACE_0500/NC_009142_0499 is certainly able to specifically recognize *dre*-like sequences and, importantly, that the sequences known to be bound by DasR^{Sc} could be used to predict the DasR regulon in *Sacch. erythraea* (see below). Taken together, all these *in silico* data show that SACE_0500/NC_009142_0499 is unquestionably the closest homologue of the GlcNAc utilization repressor DasR studied in *Streptomyces* spp. and will be named DasR^{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_0363/NC_009142_0363 (putative secreted β -*N*-acetylglucosaminidase) and SACE_5287/NC_009142_5219 (putative chitinase family GH18-C) (Table 1).

The upstream regions of these TUs (350 bp fragments) were incubated with the pure His-tagged DasR^{Sery} (Fig. S1) in order to detect possible DNA/protein interactions via gel retardation assays. EMSAs revealed that His-tagged DasR^{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^{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 nnTGGTCTAGACCAnn, which is highly similar to the anTGGTCTAGACCant 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

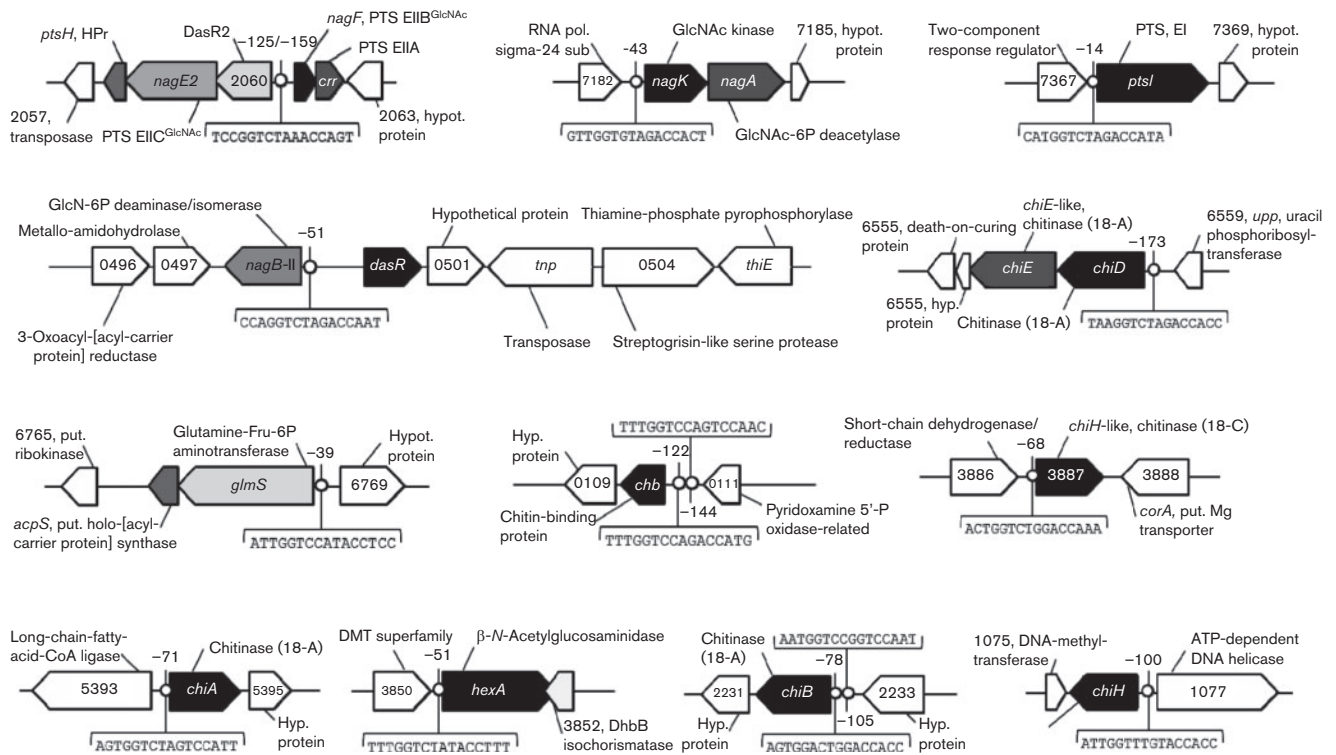


Fig. 3. Organization of DasR-target genes involved in GlcNAc and chitin metabolism in *Sacch. erythraea*. Genetic context of chitin and GlcNAc utilization genes that do possess at least one DasR responsive element (*dre*) within their upstream region. The position of the *dre* is calculated from the translational start of the downstream TU. For the known or predicted function of genes/proteins please refer to Table 1.

Sacch. erythraea, EMSA analyses revealed that GlcN-6P was able to modify the DNA-binding of DasR^{Sery} to *dre*^{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^{Sery}/*dre*^{ptsI} complex, but caused the occurrence of additional shifted bands. The multiple bands suggest that DasR^{Sery} 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*, *ptsI*, *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_6768/NC_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^{GlcNAc}) and GlcNAc catabolism (*nagKA* and *nagB-II*) in the *dasR* mutant correlates with the increased biomass accumulation of strain *Sacch. erythraea* Δ *dasR* 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_3887/NC_009142_3844 showed only enhanced expression in RNA samples from the *dasR* mutant collected at 36 h. Surprisingly, *chb*, which possesses two *dre*-like sequences in its upstream region (Fig. 3), did not show significant expression changes

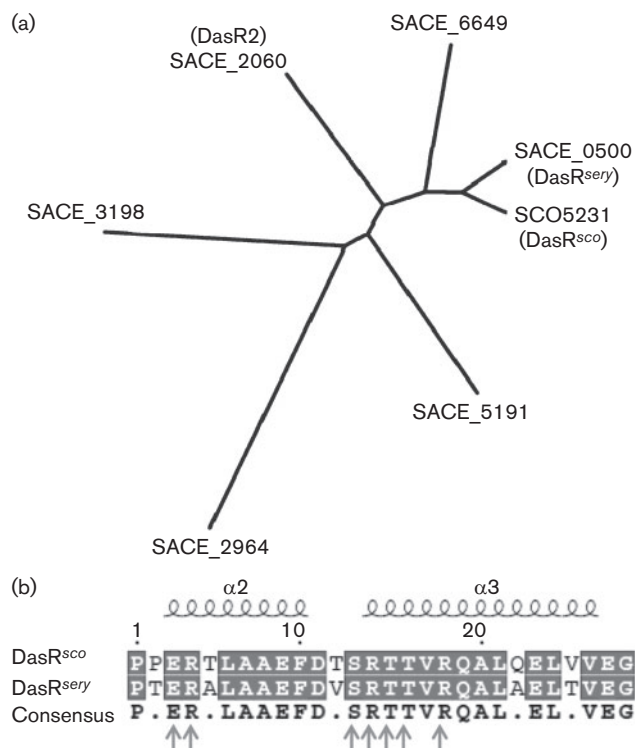


Fig. 4. *In silico* analysis of *Sacch. erythraea* DasR. (a) Phylogeny analysis of GntR/HutC subfamily regulators of *Sacch. erythraea* and DasR from *Strep. coelicolor*. Note that SACE_0500/NC_009142_0499 (DasR^{Sery}) and SCO5231 (DasR^{Sco}) emerge from a common ancestor that is different from other GntR/HutC members of *Sacch. erythraea*. (b) Alignment of helix-turn-helix motifs of SACE_0500/NC_009142_0499 (DasR^{Sery}) and SCO5231 (DasR^{Sco}). The seven residues predicted to be involved in *dre*-binding (Resch *et al.*, 2010) are conserved between *Sacch. erythraea* and from *Strep. coelicolor*.

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).

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 chitoooligosaccharide 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-acetylglucosaminidases. 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* Δ*dasR* 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-acetylglucosaminidases, which would impair feeding on chitoooligosaccharides. This is further supported by the fact that other pseudonocardiae, such as *Saccharotrix espanaensis*, *Actinosynnema mirum*, *Amycolatopsis* species, *Kutzera* 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

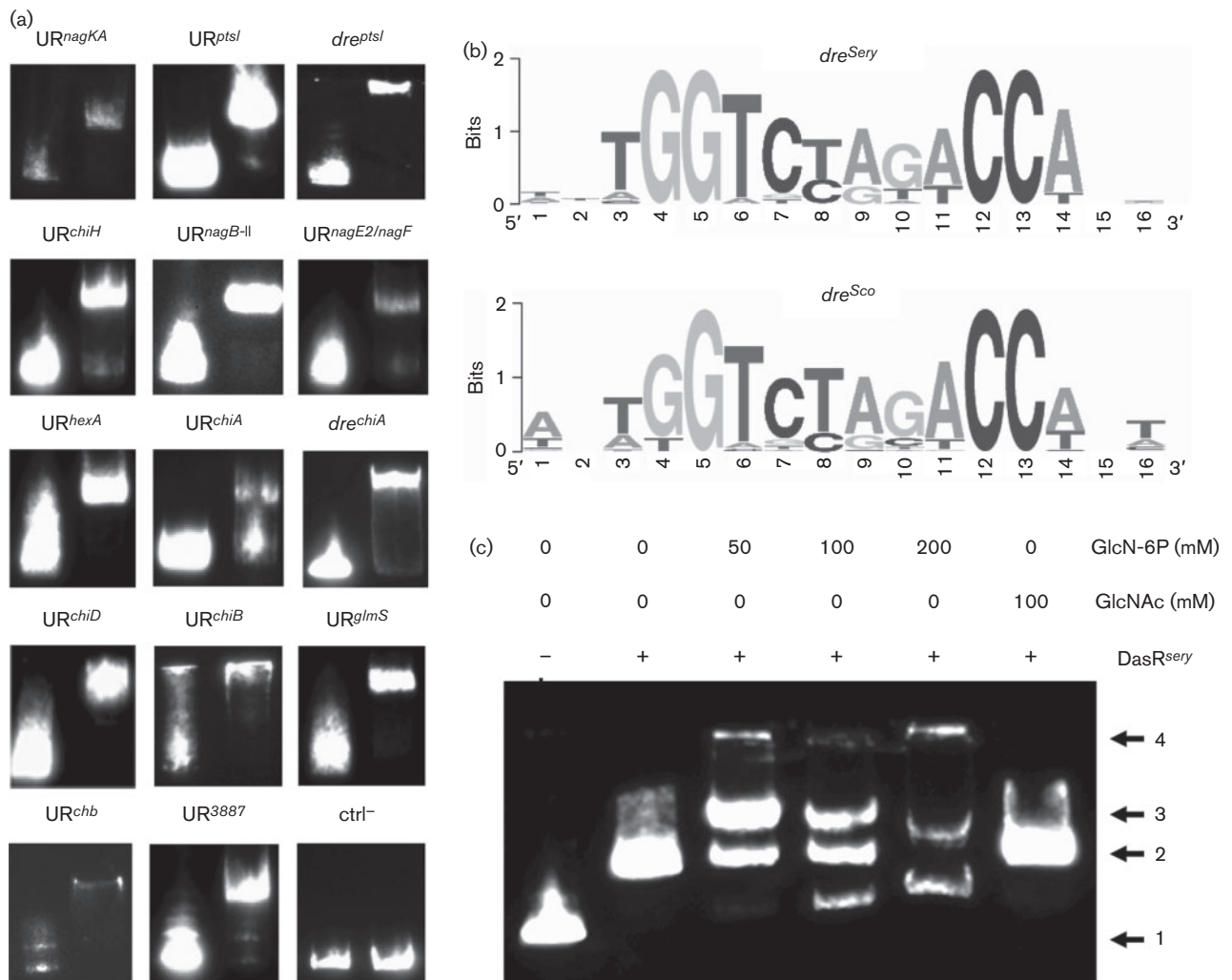


Fig. 5. DNA- and ligand-binding properties of *Sacch. erythraea* DasR. (a) EMSAs with pure His-tagged DasR^{Sery} and *dre* probes. Probes are either 350 bp fragments corresponding to the upstream region (UR, -300 to +50 nt upstream and downstream of the translational start) of genes predicted to be controlled by DasR^{Sery} 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^{Sery}. EMSAs were performed with *dre*^{ptsl} as the probe. + and - indicate the presence or absence of 1 μM His-tagged DasR^{Sery}. GlcNAc or GlcN-6P was added to the *dre*^{ptsl}-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*^{ptsl}.

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).

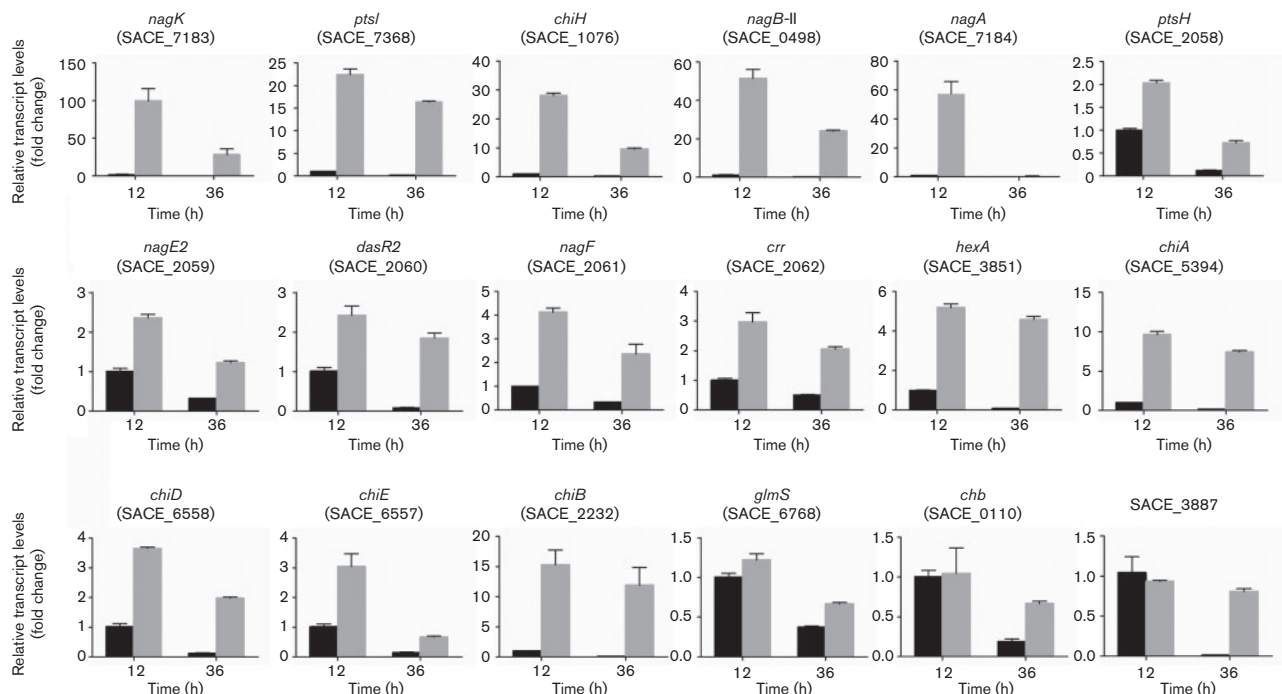


Fig. 6. Effect of *dasR* deletion on the expression of genes involved in GlcNAc and chitin metabolism in *Sacch. erythraea*. RNAs were extracted from *Sacch. erythraea* NRRL2338 (black bars) and its *dasR* mutant strain (grey bars) grown for 12 and 36 h in balanced Evans medium. Relative transcript levels 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 \pm SD from three independent experiments, each with triplicate samples, using distinct cDNA preparations for each RNA sample.

The reason why *Sacch. erythraea* cannot properly utilize chitin is probably the result of a combination of the hypotheses suggested here, although the loss of the DasABC transporter would contribute more significantly. Interestingly, *S. erythraea* presented a much better growth when spores were spotted onto an MM chitin previously 'pre-conditioned' by a culture of *Strep. coelicolor* (Fig. S3a). We speculate that the improved growth of *S. erythraea* is the consequence of the release of GlcNAc monomers from chitin by the *Strep. coelicolor* chitinases. This experiment suggests that in mixed bacterial populations where chitin is an abundant nutrient source, *Sacch. erythraea* could be considered 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 synteny 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 DasR^{Sery} and DasR^{Sco} 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

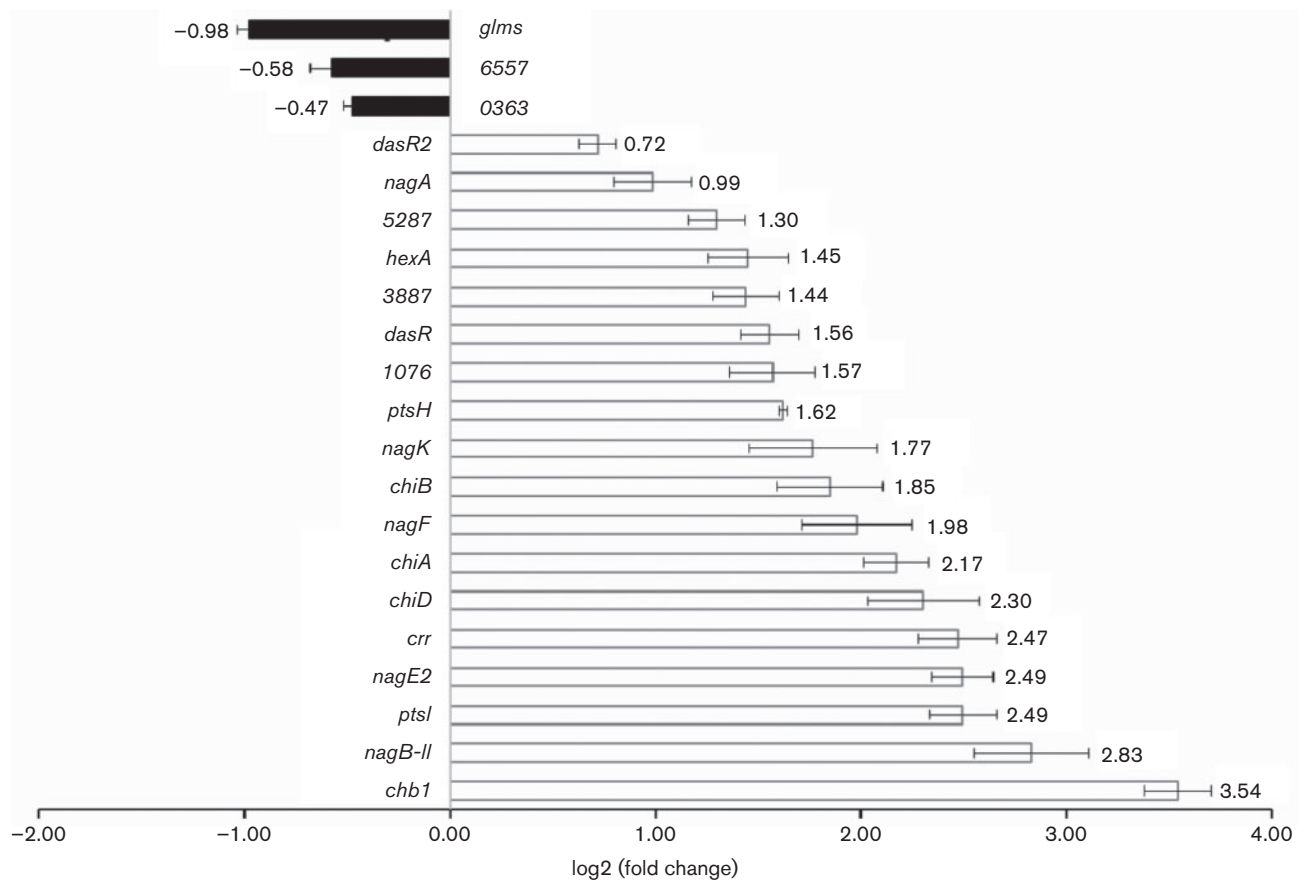


Fig. 7. Effect of GlcNAc supply on the expression of genes involved in GlcNAc and chitin metabolism in *Sacch. erythraea*. The values represent the transcriptional pattern of the GlcNAc operon and genes involved in GlcNAc metabolism. RNA was extracted 45 min after GlcNAc induction in glucose-based minimal medium MM101. Transcription values were log₂ transformed. Data shown are means of two independent experiments.

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^{Sco} does not bind to *dre* once complexed to its ligand (Rigali *et al.*, 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^{Sery}, but the DNA-binding domains would be reoriented and forced apart as described for the orthologue NagR in *B. subtilis* (Resch *et al.*, 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 *et al.*, 2010). Studies on the allosteric effectors of DasR and NagR in *Strep. coelicolor* and *B. subtilis* give contradicting results based on the methodology used for EMSAs (Gaugué *et al.*, 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^{GlcNAc}, 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 *et al.*, 2002), temporarily named here DasR2. The overall amino acid conservation between DasR^{Sery} 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/

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^{Sery} 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 *Streptomyces* (Rigali *et al.*, 2006, 2008). Indeed, *das* stands for deficient in aerial hyphae and spore formation (Seo *et al.*, 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 *Streptomyces* depending on the culture conditions (Rigali *et al.*, 2008). The preliminary observation of the *Sacch. erythraea* Δ *dasR* mutant strain grown in GlcNAc-containing media also suggests impaired development compared to the wild-type strain (Fig. S3b). Accurate description of the development of the *Sacch. erythraea* Δ *dasR* 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 *Sacch. erythraea*.

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