The enigmatic lack of glucose utilization in *Streptomyces clavuligerus* is due to inefficient expression of the glucose permease gene

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Streptomyces clavuligerus ATCC 27064 is unable to use glucose but has genes for a glucose permease (*glcP*) and a glucose kinase (*glkA*). Transformation of *S. clavuligerus* 27064 with the *Streptomyces coelicolor glcP1* gene with its own promoter results in a strain able to grow on glucose. The *glcP* gene of *S. clavuligerus* encodes a 475 amino acid glucose permease with 12 transmembrane segments. GlcP is a functional protein when expressed from the *S. coelicolor glcP1* promoter and complements two different glucose transport-negative *Escherichia coli* mutants. Transcription studies indicate that the *glcP* promoter is very weak and does not allow growth on glucose. These results suggest that *S. clavuligerus* initially contained a functional glucose permease gene, like most other *Streptomyces* species, and lost the expression of this gene by adaptation to glucose-poor habitats.

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INTRODUCTION

A total of 53 putative carbohydrate uptake genes have been detected in the genome of the model organism *Streptomyces coelicolor*. ATP-binding cassette (ABC) transport systems account for the transport of ribose, lactose, maltose, xylose and sugar alcohols (Bertram *et al.*, 2004). Glucose is transported by an H⁺–glucose symporter (van Wezel *et al.*, 2005), and comparative analysis of gene sequences suggests that galactose is transported by an Na⁺–galactose symporter of the SSS (sodium solute symporters) family, similar to that found in *Vibrio parahaemolyticus* (Bertram *et al.*, 2004). Fructose is incorporated by the phosphotransferase (PTS) system, as shown by the disruption of the *ptsH* gene (Nothaft *et al.*, 2003b). A protein, GlpF, of the major intrinsic protein family appears to be responsible for glycerol-facilitated diffusion (Bertram *et al.*, 2004; Hindle & Smith, 1994). Recently, it has been shown that *N*-acetyl-D-glucosamine (NAG) plays an important role in differentiation and antibiotic production in *S. coelicolor* (Rigali *et al.*, 2006, 2008), in which this carbon source is transported by the sugar PTS system (Nothaft *et al.*, 2003a).

Abbreviations: ABC, ATP-binding cassette; PTS, phosphotransferase.

The GenBank/EMBL/DDBJ accession numbers for the sequences of the regions upstream and downstream of *S. clavuligerus glkA* and *glcP* are FN377746 and FN377747.

The behaviour of *Streptomyces clavuligerus*, the producer of clavulanic acid and cephamycin C, in relation to carbon sources utilization is peculiar. This species is unable to grow on glucose as the sole carbon source; moreover, it does not use hexoses such as mannose, galactose or fructose, pentoses such as xylose, sugar alcohols such as mannitol or sorbitol, or disaccharides such as lactose or sucrose. Only glycerol, maltose and starch are efficiently used as carbon sources by *S. clavuligerus* (García-Domínguez *et al.*, 1989).

If the systems for carbohydrate transport are conserved in different *Streptomyces* species, *S. clavuligerus* would be defective in: (i) ABC systems for lactose, xylose or mannitol uptake; (ii) the PTS system for fructose utilization; and (iii) the symporters required for glucose and galactose incorporation. This is an enigmatic situation for a soil-dwelling actinomycete.

One gene for glucose transport (*araE*, SAV2657) is present in the *Streptomyces avermitilis* genome, and two genes, *glcP1* (SCO5578) and *glcP2* (SCO7153), with 99.9% nucleotide identity to each other, separated by 1.8 Mb in the chromosome, occur in the *S. coelicolor* genome. They encode 100% identical GlcP1 and GlcP2 proteins. Both genes are expressed in glucose-grown cells of *S. coelicolor*, as shown by RT-PCR experiments; *glcP1* is inducible by glucose while *glcP2* transcripts are almost undetectable (van Wezel *et al.*, 2005). In this work we report the effect of

Table 1. Plasmids and strains used in this work

Plasmid or strain	Relevant characteristics	Source or reference
Plasmids		
pFT256	Contains <i>glkA</i> (SCO2126) controlled by its own promoter	F. Titgemeyer, Friedrich-Alexander-Universität, Erlangen, Germany
pFT76	<i>E. coli</i> vector containing <i>glcP2</i> controlled by the <i>lacZ</i> promoter	van Wezel <i>et al.</i> (2005)
pBluescript II SK (+)	General cloning <i>E. coli</i> vector	Stratagene
pUZ8002	Conjugation helper plasmid	Paget <i>et al.</i> (1999)
pLUXAR	<i>E. coli</i> / <i>Streptomyces</i> bifunctional conjugative-integrative vector	Rodríguez-García <i>et al.</i> (2007)
pIJ699	<i>E. coli</i> / <i>Streptomyces</i> bifunctional vector	Kieser <i>et al.</i> (2000)
pIJ773	pBluescript II SK (+) containing an <i>aac3(IV)</i> -oriT cassette	Gust <i>et al.</i> (2003)
pRA	<i>E. coli</i> / <i>Streptomyces</i> bifunctional conjugative-integrative vector	This work
pRA-AB	pRA containing PCR fragments assembled with the promoter region of <i>S. coelicolor glcP1</i> and the coding region of <i>S. coelicolor glcP1</i>	This work
pRA-AD	pRA containing PCR fragments assembled with the promoter region of <i>S. coelicolor glcP1</i> and the coding region of <i>S. clavuligerus glcP</i>	This work
pRA-CB	pRA containing PCR fragments assembled with the promoter region of <i>S. clavuligerus glcP</i> and the <i>glcP1</i> gene of <i>S. coelicolor</i>	This work
pRA-CD	pRA containing PCR fragments assembled with the promoter region of <i>S. clavuligerus glcP</i> and the <i>glcP</i> gene of <i>S. clavuligerus</i>	This work
pRA-AB-K	pRA-AB containing the <i>glkA</i> gene from pFT256	This work
pRA-1	pRA containing the <i>S. clavuligerus</i> putative <i>glkA</i> activator	This work
pRA-2	pRA containing the <i>S. clavuligerus</i> putative <i>glkA</i> activator and upstream region	This work
pAMB1	<i>E. coli</i> / <i>Streptomyces</i> bifunctional vector, conjugative, replicative	This work
pAMB1-1	pAMB1-derived. Contains the <i>S. clavuligerus</i> putative <i>glkA</i> activator	This work
pAMB1-2	pAMB1-derived. Contains the <i>S. clavuligerus glkA</i> activator and upstream gene region	This work
pBRel	pFT76 vector lacking a 1.2 kb <i>Bam</i> HI fragment with most of the <i>glcP2</i> gene	This work
pKS-CD	pBluescript KS II (+) containing PCR fragments assembled with both promoter region and <i>glcP</i> gene of <i>S. clavuligerus</i>	This work
p <i>glcP</i>	<i>E. coli</i> vector containing <i>S. clavuligerus glcP</i> controlled by the <i>lacZ</i> promoter	This work
Strains		
<i>E. coli</i> DH5- α	General host strain	Hanahan (1983)
<i>E. coli</i> ET12567	Host strain for plasmid conjugation	MacNeil <i>et al.</i> (1992)
<i>E. coli</i> LM1	Glucose transport-negative mutant	Lengeler <i>et al.</i> (1981)
<i>E. coli</i> LM1[pBRel]	<i>E. coli</i> LM1 transformant carrying the empty control vector pBRel	This work
<i>E. coli</i> LM1[pFT76]	<i>E. coli</i> LM1 transformant carrying in pFT76 the <i>glcP2</i> gene controlled by the <i>lacZ</i> promoter	This work
<i>E. coli</i> LM1[p <i>glcP</i>]	<i>E. coli</i> LM1 transformant carrying in p <i>glcP</i> the <i>S. clavuligerus glcP</i> gene controlled by the <i>lacZ</i> promoter	This work
<i>E. coli</i> LR2-175	Glucose transport-negative mutant	Aulkemeyer <i>et al.</i> (1991)
<i>E. coli</i> LR2-175[pBRel]	<i>E. coli</i> LR2-175 transformant carrying the empty control vector pBRel	This work
<i>E. coli</i> LR2-175[pFT76]	<i>E. coli</i> LR2-175 transformant carrying in pFT76 the <i>glcP2</i> gene controlled by the <i>lacZ</i> promoter	This work
<i>E. coli</i> LR2-175[p <i>glcP</i>]	<i>E. coli</i> LR2-175 transformant carrying in p <i>glcP</i> the <i>S. clavuligerus glcP</i> gene controlled by the <i>lacZ</i> promoter	This work
<i>S. clavuligerus</i> ATCC 27064	Wild-type strain	ATCC
<i>S. coelicolor</i> M145	Prototroph	Kieser <i>et al.</i> (2000)
<i>S. clavuligerus</i> ::pRA	Integrative exconjugant of <i>S. clavuligerus</i> 27064 carrying the empty vector pRA	This work
<i>S. clavuligerus</i> ::pRA-AB	Integrative exconjugant of <i>S. clavuligerus</i> 27064 carrying pRA containing the <i>glcP1</i> gene of <i>S. coelicolor</i> expressed from its own promoter	This work

Table 1. cont.

Plasmid or strain	Relevant characteristics	Source or reference
<i>S. clavuligerus</i> ::pRA-AD	Integrative exconjugant of <i>S. clavuligerus</i> 27064 carrying pRA containing the <i>glcP</i> gene of <i>S. clavuligerus</i> expressed from the <i>glcP1</i> promoter of <i>S. coelicolor</i>	This work
<i>S. clavuligerus</i> ::pRA-CD	Integrative exconjugant of <i>S. clavuligerus</i> 27064 carrying pRA containing the <i>glcP</i> gene of <i>S. clavuligerus</i> expressed from its own promoter	This work
<i>S. clavuligerus</i> ::pRA-CB	Integrative exconjugant of <i>S. clavuligerus</i> 27064 carrying pRA containing the <i>glcP1</i> gene of <i>S. coelicolor</i> expressed from the <i>glcP</i> promoter of <i>S. clavuligerus</i>	This work
<i>S. clavuligerus</i> ::pRA-AB-K	Integrative exconjugant of <i>S. clavuligerus</i> 27064 carrying pRA containing the <i>glcP1</i> and <i>glkA</i> genes of <i>S. coelicolor</i> expressed from their own promoters	This work

S. coelicolor glcP1 (for glucose transport) and *glkA* (encoding the glucose kinase) genes on glucose utilization by *S. clavuligerus*. Using a combination of the *S. coelicolor* and *S. clavuligerus* glucose permease promoters coupled to either the promoterless *glcP* gene of *S. clavuligerus* or the promoterless *glcP1* gene of *S. coelicolor* we conclude that expression of *glcP* from the native *S. clavuligerus* promoter is limiting for glucose utilization.

METHODS

Bacterial strains, plasmids and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Cultures of plasmid-bearing cells were supplemented with ampicillin (50 µg ml⁻¹), chloramphenicol (25 µg ml⁻¹), kanamycin (25 µg ml⁻¹) or apramycin (50 µg ml⁻¹), as appropriate.

Glucose transport-deficient *Escherichia coli* mutants and transformants carrying the *glcP* gene were grown on MacConkey or in Voges-Proskauer medium (Difco).

To inoculate cultures of *S. coelicolor*, spores were germinated in 2 × tryptone yeast extract (TY) medium (Kieser *et al.*, 2000). *S. clavuligerus* cultures were inoculated using culture aliquots [grown in tryptone soya broth (TSB) medium] kept frozen in 20% (v/v) glycerol. *Streptomyces* strains were grown in 500 ml baffled flasks at 28 °C and 220 r.p.m.

To check the growth on different carbon sources, strains were inoculated in 50 ml of inoculum medium (IM), which contains basal medium (BM) supplemented with 0.2% L-asparagine, 1% glycerol, 0.1% NH₄Cl and 0.1% yeast extract (Aharonowitz & Demain, 1978). After 24 h, the mycelium was washed and different volumes, as appropriate, were transferred to 50 ml BM containing 0.1% NH₄Cl and 1% of the carbon source tested. Glucose solutions were treated with α-glucosidase to eliminate traces of maltose present in commercial glucose supplies. For the growth of *S. coelicolor*, BM was supplemented with the trace elements described by Chatterjee & Vining (1981).

Glucose in the culture supernatants was quantified with the Glucose Assay kit (Sigma).

Growth was determined by measuring total DNA by the diphenylamine method (Burton, 1968).

DNA manipulations. Plasmid DNA isolation, restriction endonuclease digestions, ligations and transformation of *E. coli* were

performed using standard techniques (Sambrook *et al.*, 1989). Genomic DNA preparations from *Streptomyces* were carried out according to Kieser *et al.* (2000). Non-radioactive hybridizations were performed following the protocol given in the DIG System kit (Roche), and detection was done using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

DNA sequencing. DNA sequencing of the PCR-amplified fragments was performed using a MegaBACE 500 DNA Analysis System (Amersham Biosciences) sequencer. Subclones were sequenced from universal primers, checking the information on both strands. The sequences of the regions upstream and downstream of *S. clavuligerus glkA* and *glcP* were provided by DSM (Delft, The Netherlands) and have been deposited in the EMBL database with the accession numbers FN377746 and FN377747.

RNA isolation. RNeasy Protect Bacteria reagent (Qiagen) was used for stabilization of collected samples for RNA extraction. Mycelium was treated with lysozyme (30 mg ml⁻¹) and RNeasy Mini Spin Columns (Qiagen) were used for RNA isolation according to the manufacturer's instructions, including a phenol extraction step before transferring the lysate to the column. RNA preparations were incubated with Turbo DNase (Ambion) to eliminate any chromosomal DNA contamination. RNA quantification was done with a NanoDrop ND-1000 UV-vis spectrophotometer.

PCR and non-quantitative RT-PCR analysis. Oligonucleotide primers used in this work are shown in Table 2. All PCRs were performed in a TGradient (Biometa) thermocycler using Platinum Pfx DNA polymerase (Invitrogen). The dNTP mix was prepared from individual nucleotides using a ratio of 15 A:15 T:35 G:35 C to improve amplification of high-G + C DNA content. Total DNA from *S. coelicolor* was used to amplify the *glcP1* (SCO5578) promoter with primers CRP1/CRP2 and the *glcP1*-coding region with CRP3/CRP4, whereas *S. clavuligerus* total DNA was used to amplify the *glcP* promoter using the oligonucleotide pair CRP5/CRP6 and the *glcP*-coding region with CRP7/CRP8. PCR products were subcloned into pBluescript II SK(+) and sequenced for confirmation before assembling the promoter-gene constructs for integration in *Streptomyces*.

Gene expression analysis by RT-PCR was done with the SuperScript One-Step system (Invitrogen) using 150–200 ng total RNA as template. Primers were designed to generate PCR products of approximately 500–600 bp. RT-PCR conditions were as follows: first strand cDNA synthesis, 50 °C for 30 min followed by 94 °C for 2 min; PCR amplification was performed in two phases: three cycles of 94 °C for 30 s, 68–62 °C (depending on the set of primers used)

Table 2. Oligonucleotides used in this work

Primer	Sequence (5'→3')*	Restriction site	Use
GLCP1†	GCGATGGGCGGTTTCCTG		Forward primer for <i>S. coelicolor glcP1</i> gene RT-PCR
GLCP2†	CGGGGATGGCGAAGGACAG		Reverse primer for <i>S. coelicolor glcP1</i> gene RT-PCR
GLCP5‡	GGATCGACCGGGCCAGGAAGG		Forward primer for <i>S. clavuligerus glcP</i> gene RT-PCR
GLCP6‡	CCCCGAGGAGACCCAGACCA		Reverse primer for <i>S. clavuligerus glcP</i> gene RT-PCR
RT-glK7	CCGCGGGCTATGTGGACGAC		Forward primer for <i>S. clavuligerus glkA</i> gene RT-PCR
RT-glK8	GAGACGCCGCCCGATGATGAAC		Reverse primer for <i>S. clavuligerus glkA</i> gene RT-PCR
CRP1	<u>GAATTC</u> GTTCTTCAAGACTTGAAC	<i>EcoRI</i>	Forward primer for <i>S. coelicolor glcP1</i> promoter cloning
CRP2	CATATG <u>TACTCCTCGGG</u> CTAC	<i>NdeI</i>	Reverse primer for <i>S. coelicolor glcP1</i> promoter cloning
CRP3	ACATATGGCCAGCACATC	<i>NdeI</i>	Forward primer for <i>S. coelicolor glcP1</i> gene cloning
CRP4	AGATCTGCGGGTCAGCCCATTTC	<i>BglII</i>	Reverse primer for <i>S. coelicolor glcP1</i> gene cloning
CRP5	<u>GAATTC</u> GGGGTCTCCAAGGTGATC	<i>EcoRI</i>	Forward primer for <i>S. clavuligerus glcP</i> promoter cloning
CRP6	CATATGA <u>ACTCCTGGGG</u> CCG	<i>NdeI</i>	Reverse primer for <i>S. clavuligerus glcP</i> promoter cloning
CRP7	CATATGAGCAGCAGGACTCCC	<i>NdeI</i>	Forward primer for <i>S. clavuligerus glcP</i> gene cloning
CRP8	ACGGACGAGTTCTGACGC		Reverse primer for <i>S. clavuligerus glcP</i> gene cloning

*Restriction sites added in the 5' region of cloning oligonucleotides are underlined.

†Primers GLCP1/GLCP2 are specific for *glcP1* and do not amplify the *glcP* gene.

‡Primers GLCP5/GLCP6 are specific for *glcP* and do not amplify the *glcP1* gene.

for 30 s, 72 °C for 40 s and 20–35 cycles of 94 °C for 30 s, 66–58 °C (depending on the set of primers) for 30 s and 72 °C for 40 s. Systematically, to compare non-saturated amplification products, a decreasing number of PCR cycles were performed. Negative controls were carried out with each set of primers and Platinum *Taq* DNA polymerase (Invitrogen) to verify the absence of contaminating DNA in the RNA preparations.

Glucose kinase assays. *S. clavuligerus* was grown in TSB medium [Trypticase (30 g l⁻¹) in soy broth] and *S. coelicolor* in a mixture of TSB and yeast extract/malt extract (YEME) media (40:60 %) (Kieser *et al.*, 2000) for 24 h, and the mycelium was centrifuged and transferred to starch and asparagine (SA) medium (Aidoo *et al.*, 1994) supplemented with glucose (1 %) under vigorous shaking for 10 h. Cells were harvested and washed twice, and 1 g mycelium was suspended in 50 mM triethanolamine, pH 7.4, with Complete Protease Inhibitor Cocktail (Roche). Mechanical disruption was performed with a FastPrep system (Thermo Scientific), cell debris was removed by centrifugation and the supernatant was filtered through a PD-10 desalting column (GE Healthcare).

Glucose kinase activity was measured spectrophotometrically by monitoring the formation of NADPH in a glucose-6-phosphate dehydrogenase coupled reaction, as described by García-Domínguez *et al.* (1989). Enzyme activity is expressed as units, where one unit is the amount of enzyme that produces 1 nmol NADPH min⁻¹.

Construction of plasmids. pRA is an *EcoRV*-digested pLUXAR recircularized. pAMB-1 is pIJ773 carrying a 4.9 kb *HindIII* fragment from pIJ699.

The *S. clavuligerus* putative *glkA* activator and the ORF upstream were subcloned in a 2096 bp *NsiI*–*BclI* fragment in the single-copy integrative pRA plasmid digested with *EcoRV*, to give pRA-2. From pRA-2 a 926 bp *XmnI*–*EcoRI* fragment containing only the putative *glkA* activator was rescued and subcloned in *EcoRV*/*EcoRI*-digested pRA to obtain pRA-1. The same 2096 and 926 bp DNA fragments were subcloned in the multicopy plasmid pAMB1 linearized with *EcoRV* to get pAMB1-2 and pAMB1-1.

The plasmids harbouring PCR fragments with *S. clavuligerus* or *S. coelicolor* glucose permease promoters or coding regions were used

to assemble different arrangements of promoter and gene by means of the restriction sites included in the oligonucleotides designed. These fragments were then subcloned in *EcoRI*/*BamHI*-digested pRA to give: (i) pRA-AB (A corresponds to the promoter and B to the ORF in *S. coelicolor*), in which in a 1617 bp fragment the *S. coelicolor glcP1* promoter controls the *S. coelicolor glcP1* gene; (ii) pRA-AD, in which in a 1685 bp fragment the *S. coelicolor glcP1* promoter (A) controls the *S. clavuligerus glcP* gene (D); (iii) pRA-CB, in which in a 1633 bp fragment the *S. clavuligerus glcP* promoter (C) controls the *S. coelicolor glcP1* gene (B); and (iv) pRA-CD, in which in a 1701 bp fragment the *S. clavuligerus glcP* promoter (C) controls the *S. clavuligerus glcP* gene (D) (see Fig. 5).

To obtain pRA-AB-K, a 1 kb DNA fragment isolated from pFT256 carrying the *S. coelicolor glkA* gene expressed from its own promoter, was ligated into the *EcoRV* site of pRA-AB.

Plasmid DNA was introduced into *Streptomyces* by conjugation using *E. coli* ET12567[pUZ8002] as the donor strain. Transconjugants were isolated on mannitol–soya medium (Kieser *et al.*, 2000) and selected with apramycin. The integration of pRA-derived plasmids or the presence of the autonomous pAMB1-derived plasmids was checked by total DNA hybridization or by plasmid recovery.

Plasmid p*glcP* was constructed to check the functionality of *S. clavuligerus* glucose permease. A 1.5 kb *SacI*–*XbaI* fragment containing the *glcP*-coding region and 25 nt upstream was obtained from pKS-CD and ligated downstream of the *lacZ* promoter in *SacI*/*HindIII*-digested pFT76. The control plasmid pBRel was constructed by deletion of a 1.2 kb internal fragment of *glcP2* in *BamHI*-digested pFT76.

RESULTS

S. clavuligerus contains a *glkA* gene and has normal glucose kinase activity

To understand the lack of glucose utilization by *S. clavuligerus* we searched for a glucose kinase gene (*glkA*) in this organism. An *S. clavuligerus glkA* gene was detected

by hybridization of digested total DNA with a 1 kb probe containing the *S. coelicolor glkA* gene (Angell *et al.*, 1992). The sequence of a hybridization-positive 4.4 kb *Bst*EII DNA fragment revealed a gene organization similar to that of *S. coelicolor*, *S. avermitilis* and *Streptomyces griseus* (Fig. 1a). The *glkA* gene encodes a 313 amino acid protein (M_r 32 273, deduced pI 5.8) of the ROK family, 81% identical in amino acid sequence to the *S. coelicolor* homologue (Fig. 1b).

The *glkA* gene is located downstream of a gene (*orf2*) encoding an ion transporter ATPase, and of a gene (*orf3*) for a putative glucose kinase activator (with 57.6% identity to SCO2127) (Guzmán *et al.*, 2005). Downstream of *glkA* and separated by 679 nt is a gene (*orf5*) encoding a product of unknown function.

The *in vitro* glucose kinase activity of *S. clavuligerus* was determined in three different experiments and compared with that of *S. coelicolor*. Both strains were grown in SA medium supplemented with 1% glucose. A specific activity of 23.2 ± 3.2 mU (mg protein) $^{-1}$ was found in *S. coelicolor* cell extracts, while the mean specific glucose kinase activity in *S. clavuligerus* was 17.25 ± 2.3 mU (mg protein) $^{-1}$.

Transformant *S. clavuligerus* [pFT256] carrying the *S. coelicolor glkA* gene expressed from its own promoter was also not able to use glucose, although the glucose kinase activity in the transformant was 19.7 ± 1.6 mU (mg protein) $^{-1}$.

The SCO2127 gene of *S. coelicolor* has a positive effect on glucose uptake and glucose kinase activities (Guzmán *et al.*, 2005), possibly due to transcription activation. Therefore,

Fig. 1. Characteristics of the glucose kinase gene of *S. clavuligerus*. (a) Organization of a *Bst*EII DNA fragment containing the incomplete *orf1* (encoding a putative cyclase-dehydratase), *orf2* (encoding an ion transport ATPase), *orf3* (encoding a putative *glkA* activator), *glkA* (*orf4*) and an incomplete *orf5* (encoding a protein of unknown function). The *glkA* gene is named SCCG_05538.1 in the *S. clavuligerus* Broad Institute Project Sequence. *Bs*, *Bst*EII; *S*, *Sal*I; *Ns*, *Nsi*I; *X*, *Xmn*I; *B*, *Bcl*I. Horizontal bars indicate the DNA fragments used in the construction of pRA-1, pRA-2, pAMB1-1 and pAMB1-2. (b) Comparison of *S. clavuligerus* and *S. coelicolor* GlkA. The characteristic set of cysteines required for activity (Mesak *et al.*, 2004) is indicated with a horizontal line. Identical amino acids are indicated with asterisks. (c) Expression of *glkA*, detected by RT-PCR, in strains carrying different fragments containing *orf3* in single-copy integrated form (*S. clavuligerus*::pRA-1, *S. clavuligerus*::pRA-2), in a multicopy plasmid (*S. clavuligerus*[pAMB1-1], *S. clavuligerus*[pAMB1-2]) and in negative controls (*S. clavuligerus*::pRA, *S. clavuligerus*[pAMB1]). RNA was obtained in SA medium under control conditions (0) and 3 h (G) after glucose supplementation.

S. clavuligerus strains carrying *orf3*, the SCO2127 homologous gene, in a mono-copy integrative plasmid (named pRA-1 and pRA-2), or in a multi-copy plasmid (named pAMB1-1 and pAMB1-2) along with their controls (pRA and pAMB1) were constructed. When the RNA of these strains, grown in SA medium or SA supplemented with glucose, was tested by RT-PCR, they showed the same amplification intensity for the *glkA* gene (Fig. 1c). This indicates that *S. clavuligerus orf3* is not a *glkA* activator and that glucose does not stimulate *glkA* transcription.

The *S. clavuligerus glcP* gene encodes a glucose permease functional in *E. coli*

Labelled glucose is barely incorporated in *S. clavuligerus* (García-Domínguez *et al.*, 1989). To test whether a glucose permease gene exists in *S. clavuligerus*, total DNA was hybridized with a 1.4 kb DNA fragment containing the whole *S. coelicolor glcP2* gene. A clear signal of 6 kb was found in *Bst*EII-digested DNA; therefore, the genome of *S. clavuligerus*, available at DSM (Delft, The Netherlands), was searched for a gene homologous to *S. coelicolor glcP1* or *glcP2*. One single glucose permease gene located downstream of a chromosome segregation gene (*orf6*) was found, while in the opposite orientation, genes for a putative acetyltransferase (*orf8*) and a putative docking protein (*ftsY*) were present (Fig. 2a).

The *S. clavuligerus* glucose permease gene was named *glcP* to differentiate it from the *S. coelicolor glcP1* and *glcP2* genes. It encodes a protein with 80–81% identity to *S. coelicolor GlcP1*, *S. avermitilis AraE* and *S. griseus SGR1900* (Fig. 2b). The protein (M_r 50 539, pI 8.4), contains 12 membrane-spanning segments as found in other H^+ -glucose symporters.

Two glucose transport-negative mutants, *E. coli* LM1 and *E. coli* LR2-175 (Aulkemeyer *et al.*, 1991; Lengeler *et al.*, 1981), were transformed with plasmid p*glcP*, in which *S. clavuligerus glcP* is expressed from the *lacZ* promoter, and with plasmids pFT76 (containing *S. coelicolor glcP1*) and pBRel, as positive and negative controls, respectively. The strains were streaked on MacConkey agar and on MacConkey agar supplemented with 50 mM glucose. Transformants *E. coli* LM1[p*glcP*] and *E. coli* LR2-175[p*glcP*] were able to use glucose, as indicated by the yellow halo formed around the patches (Fig. 3a), whereas the parental strains and transformants *E. coli* LM1[pBRel] and *E. coli* LR2-175[pBRel] did not produce yellow zones. In addition, the strains were grown for 48 h in Voges–Proskauer medium and supplemented with methyl red. The culture broth of *E. coli* LR2-175[pFT76] and *E. coli* LR2-175[p*glcP*] turned from slightly yellow to intense red (Fig. 3b), indicating acidification due to glucose fermentation, while the parental mutant and control negative strain retained the pale yellow colour, confirming that they are not able to use glucose. This indicates that *S. clavuligerus* glucose permease is functional when expressed in *E. coli*.

glcP is weakly expressed in *S. clavuligerus*

In *S. coelicolor* the essential glucose permease is *glcP1*, while the duplicate *glcP2* gene can be deleted without a significant effect on glucose utilization. The two genes have substantial differences in their 5'-upstream regions. When compared with the homologous regions in *S. avermitilis araE* and *S. clavuligerus glcP*, an overall similarity is found between *glcP1* and *araE* upstream regions. The -10 (TAGTCT) and -35 (TTGACT) sequences of *glcP1* (van Wezel *et al.*, 2005) are perfectly conserved in *araE*. However, the homology is very low with the promoter regions of *S. coelicolor glcP2* (results not shown) or *S. clavuligerus glcP*, in which the -10 and -35 regions are difficult to recognize (see Fig. 2c).

The *glcP* gene in *S. clavuligerus* is 161 nt downstream of *orf6* and 55 nt upstream, and in the opposite sense to *orf8* (Fig. 2a). RT-PCR analysis was done using RNA isolated from *S. clavuligerus::pRA* (control strain carrying the integrated pRA empty plasmid) grown in: (i) BM plus glycerol, (ii) BM plus glucose (Fig. 4, upper panel) or (iii) SA, and (iv) SA plus glucose (Fig. 4, lower panel). To discriminate strand-specific transcription, the experiment was performed in two steps: (i) mRNA retrotranscription to obtain cDNA was done using primers GLCP5 or GLCP6 separately, and (ii) the second oligonucleotide (either GLCP6 or GLCP5, respectively) was added later for the PCR process. When the cDNA was obtained using GLCP6, complementary to the *glcP* mRNA (Fig. 4, lanes 3 and 7), the yield of amplified product was barely detectable and clearly lower than when the retrotranscription was done with the GLCP5 primer (Fig. 4, lanes 2 and 6). These results indicate that a very weak transcription of *glcP* occurs but that most of the signal in that region is due to an antisense transcript, probably originating in *orf8*. The residual expression of *glcP* is not affected by the presence of glucose in the cultures (Fig. 4, lanes 3 and 7).

Construction of *S. clavuligerus* recombinant strains able to use glucose

The promoter regions of *glcP1* from *S. coelicolor* and *glcP* from *S. clavuligerus*, as well as *S. coelicolor glcP1*- and *S. clavuligerus glcP*-coding sequences, were amplified by PCR. The four PCR products were assembled in the four combinations (two promoters $\times$ two ORFs) indicated in Fig. 5a and subcloned in the integrative single-copy plasmid pRA in such a way that each gene was expressed either from its own promoter (constructions pRA-AB and pRA-CD, where the last two letters refer to the DNA fragments combined) or from the promoter of the homologous gene (constructions pRA-AD and pRA-CB). *S. clavuligerus* exconjugants carrying the integrated plasmids were grown in BM medium with glucose or glycerol as sole carbon source. All the strains grew on glycerol but only *S. clavuligerus::pRA-AB* and *S. clavuligerus::pRA-AD*, expressing *S. coelicolor glcP1* and *S. clavuligerus glcP*, respectively, from the *S. coelicolor glcP1* promoter, grew on

Fig. 2. Characteristics of the glucose permease gene (*glcP*) of *S. clavuligerus*. (a) Organization of the genome in the region around *glcP*: incomplete *orf6* (encoding a chromosome segregation protein), *glcP* (*orf7*), *orf8* (encoding an acetyltransferase) and incomplete *ftsY*. The *glcP* gene is named SCCG_07234.1 in the *S. clavuligerus* Broad Institute Project Sequence. *S*, *Sal*; *B*, *Bcl*I; *N*, *Nco*I; *Bs*, *Bst*EI; *E*, *Eco*RI. (b) Comparison of the proteins encoded by *S. clavuligerus glcP* and *S. coelicolor glcP1*. The 12 transmembrane motifs predicted for GlcP are indicated with horizontal lines. (c) Comparison of the promoter-containing intergenic regions upstream of *araE* from *S. avermitilis*, *glcP1* from *S. coelicolor* and *glcP* from *S. clavuligerus*. The -10 and -35 regions and the transcription start point of the *S. coelicolor glcP1* promoter (van Wezel *et al.*, 2005) and the putative homologous regions in the *S. avermitilis araE* promoter are underlined.

Fig. 3. Complementation of glucose transport-negative *E. coli* strains by *S. clavuligerus glcP*. (a) MacConkey agar plates without (left) and supplemented with 50 mM glucose (right). (b) Liquid Voges-Proskauer with methyl red. *E. coli* strains: (1) DH5- α , (2) LM1, (3) LM1[pFT76], (4) LM1[pBRel], (5) LM1[p*glcP*], (6) LR2-175, (7) LR2-175[pFT76], (8) LR2-175[pBRel], (9) LR2-175[p*glcP*].

(Fig. 6a). The good growth of *S. clavuligerus*::pRA-AB-K on glucose shows that the combination of *glcP1* and *glkA* increases the growth of the culture (Fig. 6b, left panel).

Studies of glucose utilization by the exconjugants (Fig. 6b, right panel) indicate that *S. clavuligerus*::pRA (control

strain) did not consume glucose, but *S. coelicolor* (not shown) and *S. clavuligerus* exconjugants carrying one additional copy of the glucose permease genes consumed glucose to different degrees. Glucose depletion was faster in *S. clavuligerus*::pRA-AB-K than in *S. clavuligerus*::pRA-AB (Fig. 6b, right panel), reflecting the cooperative effect of the *glcP1* and *glkA* genes, while *S. clavuligerus*::pRA-AD, expressing the native *glcP* gene from the *glcP1* promoter, showed only 30 % glucose utilization at 96 h (not shown). The glucose consumption rates correlate well with the previously observed growth rates for the different strains (Fig. 6b).

It is interesting that *S. clavuligerus*::pRA-AB-K shows good growth in glucose but is not able to use lactose, sucrose, mannitol, galactose or fructose (1 %) as sole carbon source, confirming that GlcP1 is a glucose-specific transporter (see Discussion).

The expression of the heterologous *glcP1* gene from its own promoter (in *S. clavuligerus*::pRA-AB) and from the *glcP* promoter (in *S. clavuligerus*::pRA-CB, which does not grow on glucose) was compared using the *glcP1*-specific oligonucleotides GLCP1/GLCP2. As observed in Fig. 6(c), no amplification of the *glcP1* transcript occurred in the negative control *S. clavuligerus*::pRA (lane 1) due to the specificity of the primers. In *S. clavuligerus*::pRA-AB (lanes 3), a clear signal was observed for *glcP1* that was slightly less intense than that observed for *S. coelicolor* M145 (lane 2). Amplification of the *glcP1* transcript in *S. clavuligerus*::pRA-CB (lanes 4) was very weak when compared with that of *S. clavuligerus*::pRA-AB (lanes 3), confirming the low efficiency of the *glcP* promoter, but was still perceptible when compared with the negative control *S. clavuligerus*::pRA (lane 1). In this figure it can be seen that glucose does not stimulate transcription of the *glcP1* gene.

In summary, the lack of glucose utilization in *S. clavuligerus* can be complemented using the *glcP1* gene of *S. coelicolor* or, simply, the *glcP1* promoter coupled to the *S. clavuligerus* promoterless *glcP* gene.

Fig. 4. Transcription of *S. clavuligerus glcP*. Amplification of the *S. clavuligerus*::pRA *glcP* gene from RNA obtained in BM medium supplemented with either glycerol (BM+Gly) or glucose (BM+G) (upper panel), or in SA medium and SA supplemented with glucose (SA+G) (lower panel). Lanes: 1 and 5, one-step RT-PCR using oligonucleotides GLCP5 and GLCP6; 4 and 8, PCR negative control with the same oligonucleotides; 2 and 6, PCR performed using cDNA obtained with GLCP5 in the reverse-transcription step (see scheme at the right side); 3 and 7, PCR performed using cDNA obtained with GLCP6 in the reverse-transcription step.

DISCUSSION

Glucose utilization in *Streptomyces* species depends on two key proteins, a glucose permease and a glucose kinase. *S. coelicolor* glucose kinase formation is constitutive, but its activation is mediated by a glucose-dependent post-translational modification (van Wezel *et al.*, 2007). *S. clavuligerus* contains a constitutively expressed *glkA* gene, and shows glucose kinase activity 25 % lower than the activity found in *S. coelicolor*. Higher levels of glucose kinase activity, obtained by expression of the *S. coelicolor glkA* gene in *S. clavuligerus*, did not confer the ability to grow on glucose upon the *S. clavuligerus* transformants. The same results were obtained by increasing the copy number of *orf3*, the homologous gene to *S. coelicolor* SCO2127. This gene has been reported to stimulate

Fig. 5. Assembly of *S. clavuligerus* and *S. coelicolor* promoters and glucose permease genes. (a) The *glcP1* gene of *S. coelicolor* and the *glcP* gene of *S. clavuligerus* with their promoters are shown above. The different promoters and genes assembled in plasmid pRA give the constructs pRA-AB, pRA-AD, pRA-CD and pRA-CB, shown below. (b) Growth of *S. clavuligerus*::pRA (Δ), *S. clavuligerus*::pRA-AB (●), *S. clavuligerus*::pRA-AD (○), *S. clavuligerus*::pRA-CD (□) and *S. clavuligerus*::pRA-CB (■) on BM medium supplemented with either 1% (v/v) glycerol (left panel) or glucose (right panel).

transcription of the glucokinase gene in *Streptomyces peucetius* (Guzmán *et al.*, 2005).

S. coelicolor has two genes, *glcP1* and *glcP2* (probably originating by gene duplication), to transport glucose (van Wezel *et al.*, 2005). Expression of *glcP1* is essential for growth on glucose, while *glcP2* can be deleted without appreciable effects (van Wezel *et al.*, 2005). *S. clavuligerus*, like several other *Streptomyces* species, contains a single glucose permease gene (*glcP*), encoding a protein similar to those of *S. coelicolor* (80% identical). The *glcP2* promoter region, as well as the *S. clavuligerus* *glcP* promoter, differ significantly from those of *glcP1* of *S. coelicolor* and the homologous *S. avermitilis* *araE* gene.

In this work we demonstrate that the native *S. clavuligerus* *glcP* promoter has very low expression. The residual transcription level, originating from the *glcP* promoter, is not sufficient for glucose utilization. A similar situation occurs with the *S. coelicolor* *glcP2* promoter. This gene (*glcP2*) is able to confer glucose utilization upon *S. clavuligerus* when expressed from the tetracycline-induced *tcp830* promoter (results not shown), indicating that the lack of function of *glcP2* in *S. coelicolor* is, again, due to poor expression from an inefficient promoter. The similarity with the lack of expression of the second glucose permease gene of *S. coelicolor* further supports this hypothesis. It is likely that the redundancy of identical *glcP1* and *glcP2* genes in *S. coelicolor* has favoured the decay of expression of *glcP2* in this actinomycete.

S. clavuligerus glucose permease is functional, as shown by the growth on glucose of *S. clavuligerus*::pRA-AD. This was confirmed by complementation of *E. coli* glucose transport-negative mutants with *glcP* expressed from the *lacZ* promoter.

Carbon catabolite regulation in *S. coelicolor*, in contrast to the regulation mechanism in enterobacteria (Görke & Stülke, 2008), appears to be exerted through the constitutive glucose kinase (GlcA) (Angell *et al.*, 1992, 1994). Proteins GlcA and GlcP have been proposed to form an active complex, GlcA–GlcP, to internalize and phosphorylate glucose (van Wezel *et al.*, 2007). Our results support this proposal. The best growth of *S. clavuligerus* on glucose was obtained when the homologous *glcP1* and *glcA* genes, both from *S. coelicolor*, were introduced into *S. clavuligerus*; however, when one glucose permease of *S. coelicolor* was combined with the glucose kinase of *S. clavuligerus* the growth and glucose utilization rate were lower.

S. clavuligerus appears to have adapted to grow on glycerol, maltose or even amino acids as carbon sources, losing the ability to utilize other carbon sources. Introduction of the *S. coelicolor* *glcP1* gene results in utilization of glucose but not of other hexoses. This provides conclusive evidence that *glcP*, the direct orthologue of *glcP1* of *S. coelicolor*, indeed encodes the glucose-specific transporter of *S. clavuligerus*. The lack of utilization of several other sugars in *S. clavuligerus* suggests that this actinomycete is defective in several sugar transporters. Even if the genes

Fig. 6. Effect of *S. coelicolor glcP1* and *glkA* genes on the growth of *S. clavuligerus*. (a) Plasmid pRA-AB-K used to introduce *glkA*-*glcP1* in *S. clavuligerus*. (b) Growth of *S. clavuligerus*::pRA (▲), *S. clavuligerus*::pRA-AB-K (□) and *S. clavuligerus*::pRA-AB (○) in glucose-supplemented BM medium (left panel), and glucose utilization by the same *S. clavuligerus* exconjugants (right panel). (c) Heterologous expression of *glcP1* controlled by either the *S. coelicolor glcP1* or the *S. clavuligerus glcP* promoter, tested by RT-PCR. RNA obtained in SA medium at 0 and 9 h (G) after glucose supplementation. (1) *S. clavuligerus*::pRA, (2) *S. coelicolor*, (3) *S. clavuligerus*::pRA-AB, (4) *S. clavuligerus*::pRA-CB.

encoding those transporters are expressed, lack of interaction of the modified proteins with other proteins in the membrane or in the cytoplasm may explain the lack of growth on those carbon sources.

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