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The Phosphotransferase System of *Streptomyces coelicolor* Is Biased for *N*-Acetylglucosamine Metabolism

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Mutation of the *crr-ptsI* gene locus revealed that *Streptomyces coelicolor* uses the phosphotransferase system (PTS) for *N*-acetylglucosamine uptake. *crr*, *ptsI*, and *ptsH*, which encode the three general PTS phosphotransferases, are induced by *N*-acetylglucosamine but not by other PTS substrates. Thus, the *S. coelicolor* PTS is biased for *N*-acetylglucosamine utilization, a novel feature that distinguishes this PTS from others.

The bacterial phosphotransferase system (PTS) is a multifaceted system that is required for carbohydrate uptake, carbon catabolite repression, and chemotaxis (19). In addition, there are reports that indicate a linkage between C metabolism and other cellular processes, for example, nitrogen fixation, stress response, starvation, and pathogenicity, via the PTS (5, 9, 20, 27). While PTS research is quite advanced in gram-negative and low-GC gram-positive bacteria, knowledge about it is limited in high-GC gram-positive bacteria, to which the antibiotic-producing soil bacterium *Streptomyces coelicolor* belongs (15, 16). Analysis of *ptsH*, which encodes the general PTS phosphotransferase HPr, revealed that *S. coelicolor* uses the PTS to internalize fructose, but no role of the PTS in carbon catabolite repression could be demonstrated (2, 14, 17). In silico analysis of the genome led to the identification of *pts* genes that encode the general phosphotransferases enzyme I (EI) and enzyme IIA^{Crr} as well as three further PTS permeases (NagE1, NagE2, and MalX1) (16). We suggested that *N*-acetylglucosamine could be a possible substrate for NagE1 or NagE2. This is corroborated by an in vitro characterization of IIA^{Crr}, which can serve as a IIA protein of an *N*-acetylglucosamine-specific PTS (PTS^{Nag}) (7). In a recent publication, a PTS^{Nag} has been described in *Streptomyces olivaceoviridis*, in which the homologue of *nagE2* has been identified as the structural gene for enzyme II^{Nag} (29).

We present a mutational analysis of the *crr-ptsI* gene locus and demonstrate that EI and IIA^{Crr} are part of a PTS^{Nag} in *S. coelicolor*. We provide evidence that the two genes form an operon and that their expression, together with the third general gene of the PTS, *ptsH*, is induced by *N*-acetylglucosamine. The data suggest that the PTS of *S. coelicolor* is biased for *N*-acetylglucosamine metabolism, a novel feature that will be discussed.

Knockout mutation of *crr* and *ptsI*. Gene replacement plasmids pFT50 and pFT52 were constructed by several cloning steps to generate a *crr* and a *ptsI* mutant (Table 1). Protoplasts of M145 were transformed and mutants were isolated as described previously (4, 8). The resulting strain BAP2 (Δ*crr*::*aacC4*) carried a deletion in *crr* ranging from nucleotides (nt) 146 to 280, in which the apramycin gene cassette (*aacC4*) was placed. The *ptsI* gene in strain BAP3 (*ptsI*::*aacC4*) was interrupted by the apramycin gene at nt 665. Mutations were verified by PCRs that revealed the presence of *aac4* and the correct chromosomal position by the use of oligonucleotides that hybridized in *aac4* and on the chromosome just outside the recombination area (data not shown). As can be seen from the Western blots in Fig. 1, no IIA^{Crr} (Fig. 1A, lane 2) and no EI (Fig. 1B, lane 3) were detectable in cell extracts of BAP2 and BAP3, respectively. Both mutations had no polar effect on the expression of the adjacent gene, since an immunosignal for EI was present in BAP2 (Fig. 1B, lane 2) extract and vice versa (Fig. 1A, lane 3).

Phenotypes. BAP2 and BAP3 were examined regarding their growth phenotype on mineral medium (MM) agar plates (14). Both mutants could not grow on *N*-acetylglucosamine, whereas utilization of galactose, glucosamine, glucose, glutamate, glycerol, lactose, maltose, mannitol, mannose, ribose, sorbitol, sorbose, sucrose, and xylose was not affected. The *ptsI* mutant was additionally impaired in fermentation of fructose, which is in agreement with previous publications (14, 16, 17). A growth curve in MM supplemented with 0.1% Casamino Acids and either 50 mM glycerol, glucose, or *N*-acetylglucosamine was recorded as described to corroborate the *N*-acetylglucosamine-negative phenotype (14). Within the first 35 h, the strains showed similar increases in biomass (Fig. 2A). BAP2 and BAP3 then entered stationary phase when *N*-acetylglucosamine was present in the medium while the wild type continued growing for 20 h. The mutants and the wild type showed similar growth curves when glucose or glycerol served as the source of carbon (data not shown).

Transport of *N*-[^{14}C]acetyl-D-glucosamine (6.2 mCi $mmol^{-1}$) at a final concentration of 20 μM was performed (23). BAP2 and BAP3 showed no detectable transport (<10 pmol of *N*-acetylglucosamine min^{-1} mg [dry weight]$^{-1}$), while the wild type incorporated *N*-acetylglucosamine at a rate of 87 ± 5 U when grown on glycerol and 344 ± 31 U when grown on glycerol plus *N*-acetylglucosamine. Hence, under these conditions transport of *N*-acetylglucosamine was inducible by a factor of four in the wild type and was impaired in both mutants.

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
Escherichia coli		
DH5α	Recipient for cloning experiments	1
ET12567	Host for preparation of nonmethylated DNA	10
Streptomyces coelicolor		
M145 A3(2)	SCP1$^-$ SCP2$^-$; prototroph	8
BAP2	M145 Δ*crr*::*accC4* Apr^r	This study
BAP3	M145 *ptsI*::*accC4* Apr^r	This study
Plasmids		
pBluescript SK(+)	Cloning vector, ColE1 replicon; Ap^r	Stratagene
pSU2718	Cloning vector, P15A replicon; Cm^r	11
pHLW1	Plasmid with *accC4* apramycin gene; Apr^r Ap^r	U. Wehmeier
pWHM3	*E. coli-Streptomyces* shuttle vector; Ap^r Tsr^r	28
pUWL-SK+	*E. coli-Streptomyces* shuttle vector; Ap^r Tsr^r	30
pMN406	Plasmid containing *mycgfp2*$^+$; Hyg^r	This study
pFT32	2,881-bp *Eco*RV-*Pvu*II *crr* region from cosmid SC1A8A cloned into *Sma*I of pSU2718; Cm^r	This study
pFT33	*accC4* gene from pHLW1 cut with *Pvu*II, inserted into *Tth*III1 site of pFT32; Cm^r Apr^r	This study
pFT35	Overexpression vector for *ptsI* of *S. coelicolor*	14
pFT50	4.8-kb *Sbf*I-*Nhe*I *crr*::*accC4* fragment from pFT33 cloned into *Pvu*II site of pWHM3; Ap^r Apr^r Tsr^r	This study
pFT51	1.8-kb *Eco*RI-*Hin*dIII *ptsI* fragment from pFT35 cloned into *Eco*RI-*Hin*dIII of pWHM3; Ap^r Tsr^r	This study
pFT52	*accC4* gene from pHLW1 cut with *Bam*HI inserted into *ptsI* of pFT51 cut with *Bgl*II; Ap^r Tsr^r; Apr^r	This study
pFT73	*mycgfp*2$^+$ cloned into *Bam*HI-*Pst*I of pUWL-SK+; Ap^r Tsr^r	
pFT102	pFT73 with 765-bp (nt −638 to nt 127 of *crr*) *Kpn*I-*Pst*I fragment; Ap^r Tsr^r	This study
pFT103	pFT73 with 383-bp (nt −256 to nt 127 of *crr*) *Kpn*I-*Pst*I fragment; Ap^r Tsr^r	This study
pFT104	pFT73 with 184-bp (nt −57 to nt 127 of *crr*) *Kpn*I-*Pst*I fragment; Ap^r Tsr^r	This study
pFT105	pFT73 with 305-bp (nt −228 to nt 77 of *ptsI*) *Kpn*I-*Pst*I fragment; Ap^r Tsr^r	This study

[a] Ap, ampicillin, used at 100 mg/liter; Tet, tetracycline, used at 12 mg/liter; Tsr, thiostreptone; Apr, apramycin, and Cm, chloramphenicol, both used at 25 mg/liter.

PTS assays were carried out to investigate whether EI and IIA^{Crr} are directly required for PTS^{Nag} activity (17). Phosphoenolpyruvate-dependent phosphorylation of *N*-acetylglucosamine in cell extracts of the wild type increased about fivefold (from 7.3 ± 0.3 to 40.5 ± 2.1 nmol of *N*-acetylglucosamine-phosphate min^{-1} mg of protein^{-1}) when grown in the presence of *N*-acetylglucosamine (MM plus 0.1% Casamino Acids, 50 mM glycerol, ± 50 mM *N*-acetylglucosamine). This activity was absent in cell extracts of BAP2 and BAP3 but could be restored upon addition of His-tagged EI or His-tagged IIA^{Crr}, respectively.

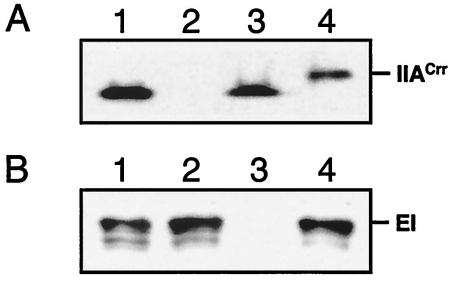

FIG. 1. Western blots of sodium dodecyl sulfate–10% polyacrylamide gels show the absence of IIA^{Crr} and EI in BAP2 and BAP3, respectively. Blotting was performed as described with polyclonal antibodies raised against His-tagged EI (Eurogentec) and His-tagged IIA^{Crr} (7). Ten micrograms of cellular protein prepared from cells grown on tryptic soy broth without dextrose or 10 ng of purified His-tagged protein was subjected to gel electrophoresis. (A) IIA^{Crr}-immunoreactive signal in cell extracts of M145 (lane 1), BAP2 (lane 2), BAP3 (lane 3), and His-tagged IIA^{Crr} (lane 4). (B) EI-immunoreactive signal in cell extracts of M145 (lane 1), BAP2 (lane 2), BAP3 (lane 3), and His-tagged EI (lane 4).

Carbon source-dependent synthesis. Since IIA^{Crr} and EI as well as the previously characterized HPr protein can be considered pleiotropically acting PTS proteins, we raised the question whether their synthesis is stimulated by growth on PTS substrates in comparison to growth on non-PTS carbon sources like glycerol and glucose. Protein levels of IIA^{Crr}, EI, and HPr were monitored by Western blotting (Fig. 3). All three proteins showed 8- to 10-fold-higher amounts in mycelia grown on *N*-acetylglucosamine than in mycelia grown on fructose, glycerol, or glucose. Interestingly, growth on the other PTS sugar, fructose, did not lead to an enhanced synthesis of the PTS proteins.

Transcription of *crr* and *ptsI*. Next, we studied transcription of the *crr-ptsI* locus. Promoter activities were examined by taking advantage of the reporter gene *mycgfp2*$^+$, a GC-rich variant of an improved *gfp* gene (24). The *mycgfp*2$^+$ gene was amplified from pMN406 by using oligonucleotides GFP1 (5′-TGCCACGGATCTGCAGGCT*TAATTAACTGA*AAGG-3′) and GFP2 (5′-CGACGGATCCGATAAAATAAAAAAGGGG-3′) introducing *Pst*I and *Bam*HI restriction sites (underlined) and stop codons in all reading frames (italics) prior to the ribosomal binding site and ligated in the *Streptomyces-Escherichia coli* shuttle plasmid pUWL-SK digested with the same endonucleases, giving plasmid pFT73 (Fig. 4A) (30). A set of transcriptional fusions of putative pro-

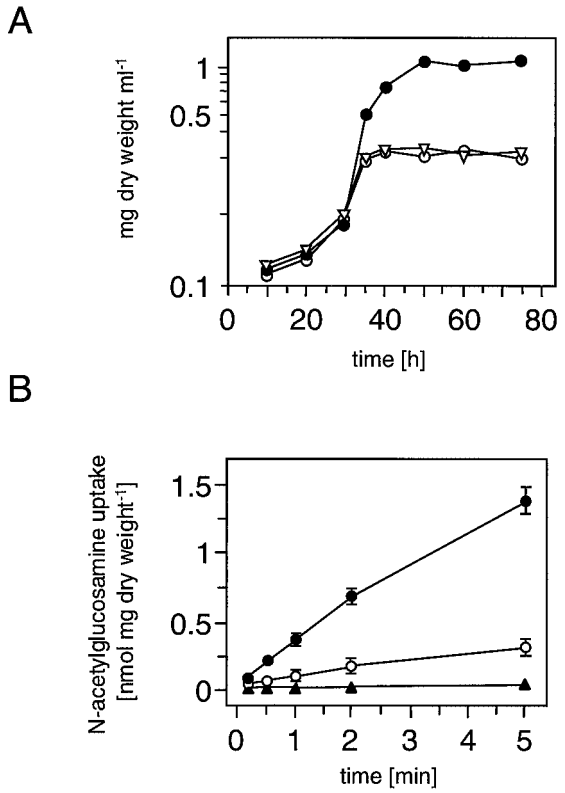

FIG. 2. *N*-Acetylglucosamine utilization and *N*-acetylglucosamine transport. (A) Growth curve of M145 (●), BAP2 (○), and BAP3 (▽) on MM supplemented with 0.1% Casamino Acids and 50 mM *N*-acetylglucosamine. (B) Time course of *N*-acetylglucosamine transport. Mycelia of M145, BAP2, and BAP3 were grown in MM supplemented with 0.1% Casamino Acids and 50 mM glycerol (○) or 50 mM glycerol plus 50 mM *N*-acetylglucosamine (●). The triangle indicates transport activity of BAP2 and BAP3 grown on glycerol with or without *N*-acetylglucosamine. Standard deviations indicated by error bars represent the means of three independent experiments.

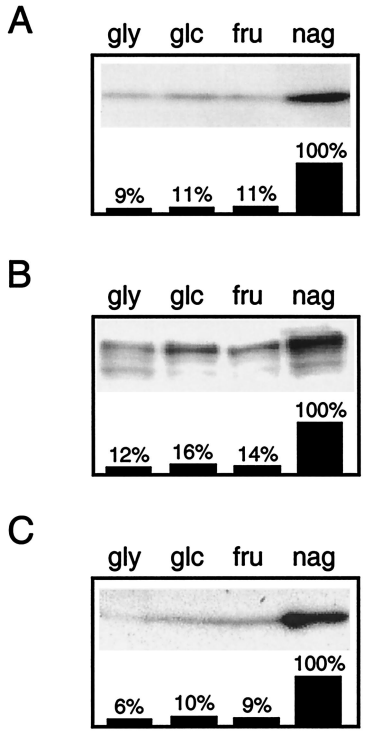

FIG. 3. Protein levels of IIA^{Crr}, EI, and HPr. Western blots of sodium dodecyl sulfate–10% polyacrylamide gels show the immunoreactive signals corresponding to IIA^{Crr} (A), EI (B), and HPr (C). Crude cell extracts were prepared from mycelia grown on MM supplemented with 0.1% Casamino Acids and 50 mM glycerol (gly), glucose (glc), fructose (fru), or *N*-acetylglucosamine (nag). In each lane 5 μg of cellular protein extract was subjected to gel electrophoresis. The bars show the signal quantification of IIA^{Crr}, EI, and HPr protein levels obtained by densitometric analysis of the gray scales with the TINA software program (version 2.08; Raytest). The signal value detected in *N*-acetylglucosamine-grown cells was set to 100%. Similar results were obtained in three independent experiments.

moter fragments was cloned into pFT73, yielding plasmids pFT102 to pFT105 (Table 1). They were transformed into the wild type. Each strain was grown on glycerol, fructose, glucose, and *N*-acetylglucosamine and subjected to green fluorescent protein fluorescence quantification. The results revealed the presence of an *N*-acetylglucosamine-dependent promoter upstream of *crr* between nt −256 and −57. This promoter was also active when the strains were grown in the presence of glycerol, glucose, and fructose. A second promoter was found to precede *ptsI*. This promoter was also inducible by *N*-acetylglucosamine.

Reverse transcription-PCR (RT-PCR) experiments were performed to corroborate these findings (Fig. 4B). RNA of *S. coelicolor* grown on MM in the presence of 0.1% Casamino Acids plus a 50 mM concentration of either glycerol, fructose, glucose, or *N*-acetylglucosamine was prepared as described previously (14). The One-Step RT-PCR kit (Qiagen) was applied according to the manufacturer's instructions with gene-specific oligonucleotides. Analysis of the mRNA of *ptsH*, *crr*, and *ptsI* and of a potential *crr-ptsI* operon transcript showed that they all were increased in RNA preparations from wild-type mycelia grown in the presence of *N*-acetylglucosamine, while RNA from fructose-, glucose-, or glycerol-grown mycelia exhibited lower amounts of transcript. Hence, the gene expression data confirmed the observed stimulation by *N*-acetylglucosamine of HPr, EI, and IIA^{Crr} synthesis and revealed that *crr* and *ptsI* constitute an operon.

In *Bacillus subtilis*, *ptsH* and *ptsI* form an operon that is constitutively expressed and that is localized downstream of *ptsG*, the gene for the glucose-specific PTS permease (6, 26). In *E. coli*, *ptsH*, *ptsI*, and *crr* form a tricistronic operon, which is regulated in a complex fashion by the globally acting transcription factors catabolite activator protein and Mlc (18). The expression varies about three- to fourfold and is stimulated either by cyclic AMP or by the presence of PTS substrates (3, 12, 21). Hence, the *N*-acetylglucosamine-specific induction of the genes of the *S. coelicolor* PTS is a novel feature that distinguishes this PTS from others.

What can be the physiological rationale for an *N*-acetylglucosamine-biased PTS? Streptomycetes are an integral part of the indigenous soil microflora. Chitin (a β-1,4-linked polymer of *N*-acetylglucosamine) and its breakdown products are commonly found in the soil. As chitin cannot directly enter the cell, it has to be degraded to chito-oligosaccharides and *N*-acetyl-

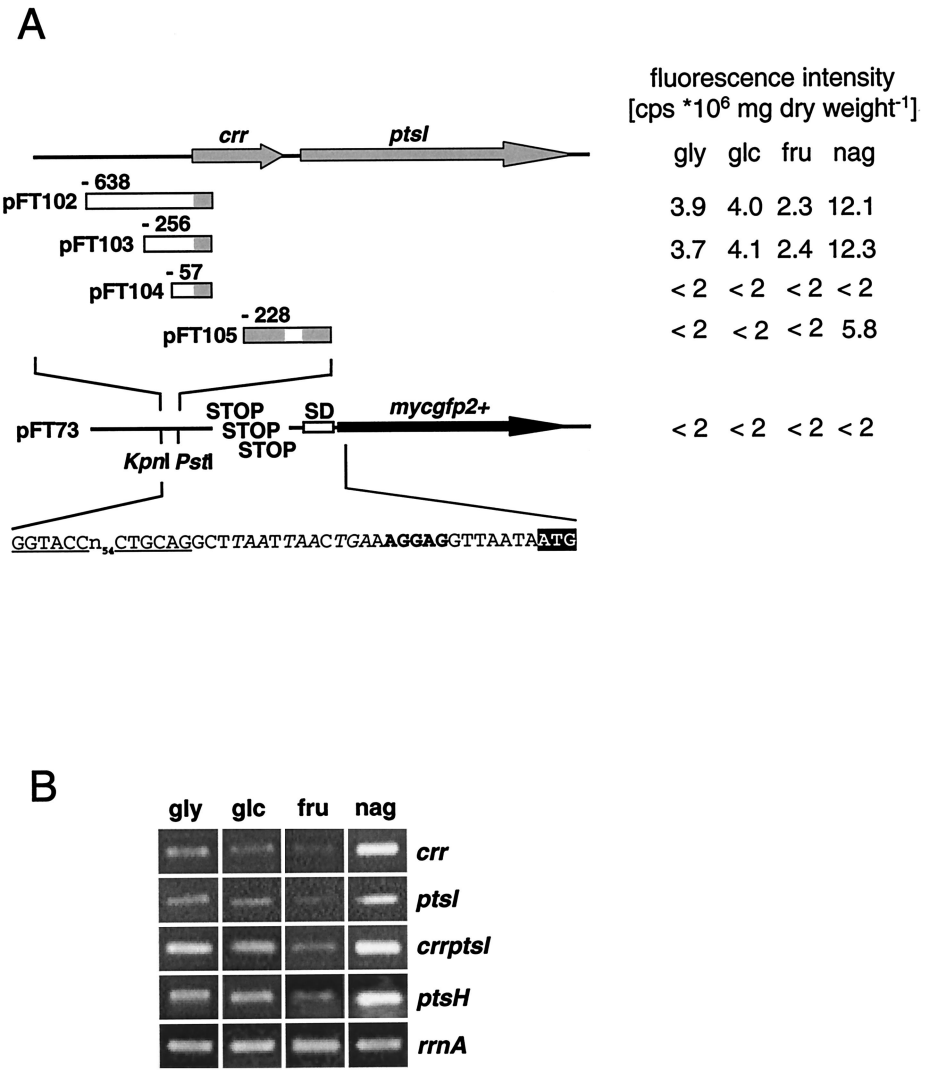

FIG. 4. Transcriptional analyses. (A) Quantification of green fluorescent protein fluorescence was carried out with mycelia grown in 30 ml of MM supplemented with 0.1% Casamino Acids and 50 mM glycerol (gly), fructose (fru), glucose (glc), or *N*-acetylglucosamine (nag) under vigorous shaking for 48 h at 28°C. Cells were harvested and washed twice in buffer A (1× phosphate-buffered saline and 0.05% Tween 80), resuspended in the same buffer, and adjusted to 2 mg (dry weight) per ml. Cells (0.5 ml) were mixed with 1.5 ml of buffer A in acrylic cuvettes (Sarstedt) and stirred to prevent sedimentation. Probes were analyzed by fluorescence spectroscopy with a Spex Fluorolog 1680. Emission spectra of *mycgfp2*$^{+}$ fluorescence were taken from 500 to 524 nm with an excitation wavelength of 485 nm and a slit width of 2 mm. cps, counts per second. (B) RT-PCR. A 1% agarose gel shows specific RT-PCR amplification products of *crr*, *ptsI*, and *ptsH* after the 27th cycle of the PCR. Total RNA was prepared from cultures grown on MM supplemented with 0.1% Casamino Acids and 50 mM glycerol (gly), glucose (glc), fructose (fru), or *N*-acetylglucosamine (nag). Oligonucleotides for RT-PCR experiments were combined as follows: RTcrr1 (22-mer) and RTcrr2 (21-mer) (nt 4 to 446 of *crr*), RTptsI1 (23-mer) and RTptsI2 (21-mer) (nt 277 to 746 of *ptsI*), RTcrrptsI1 (24-mer) and RTcrrptsI2 (24-mer) (nt 233 of *crr* to nt 119 of *ptsI*), RTptsH1 (29-mer) and RTptsH2 (25-mer) (nt 1 to nt 268 of *ptsH*), and 16SrRNA1 (21-mer) and 16SrRNA2 (21-mer) (nt 787 to nt 1313 of *rrnA*) for detection of the 16S rRNA, which served as the invariant standard.

glucosamine (22, 25). It is obvious that the genes for chitin and *N*-acetylglucosamine metabolism are coordinately regulated. We found a common *cis* element, a 12-bp palindrome that is present in one copy 122 nt upstream of *crr*, in two copies in the promoter region of *ptsH*, and in front of chitinase genes (16, 17). Transcriptional analyses of chitinase genes revealed that the *cis* element is involved in substrate induction and glucose repression (13, 22). Thus, it will be worthwhile to identify the *trans*-acting element(s) to uncover the regulation of *chi* and *pts* genes and to determine whether both pathways are subject to a common regulatory mechanism.

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