Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 2528–2532, April 1982
Biochemistry

Molecular cloning of the *crr* gene and evidence that it is the structural gene for III^{Glc}, a phosphocarrier protein of the bacterial phosphotransferase system

(diauxie/phospho*enol*pyruvate:glycose phosphotransferase system transport/regulation of sugar transport)

NORMAN D. MEADOW, DAVID W. SAFFEN, ROBERT P. DOTTIN, AND SAUL ROSEMAN

Department of Biology and the McCollum–Pratt Institute, The Johns Hopkins University, Baltimore, Maryland 21218

Contributed by Saul Roseman, January 26, 1982

ABSTRACT **Sugar substrates of the phospho*enol*pyruvate:glycose phosphotransferase system (PTS) normally prevent bacterial cells from utilizing sugars that are not substrates of this system (diauxic growth, "the glucose effect"). We have previously shown that this type of PTS-mediated repression can be completely reversed by a single mutation, designated *crr*. Two lines of evidence are presented in this report showing that *crr* is the structural gene for III^{Glc}, one of the proteins of the PTS. First, homogeneous III^{Glc} was isolated from wild-type and a crr^- mutant of *Salmonella typhimurium*, and the proteins were compared. The preparations of III^{Glc} were indistinguishable except as follows: III^{Glc} from the mutant showed only 2–3% of the activity of the wild-type III^{Glc} in its ability to act as a phosphocarrier protein in the *in vitro* phosphorylation of methyl α-glucoside. In addition, under certain conditions, the two proteins exhibited different behavior on gel filtration columns and in polyacrylamide gel electrophoresis. The second line of evidence was obtained by cloning the *Escherichia coli crr* gene, which has an estimated minimum length of 0.6 kilobase, into a high-copy-number plasmid as part of a 1.3-kilobase fragment. The plasmid transforms *E. coli* crr^- to crr^+ strains and simultaneously directs the synthesis of III^{Glc}.**

The phospho*enol*pyruvate:glycose phosphotransferase system (PTS) is a complex system of interacting proteins with a variety of physiological functions in bacterial cells (1). One such function is the translocation of its sugar substrates across the cytoplasmic membrane with concomitant phosphorylation of the sugar; phospho*enol*pyruvate (*P-e*Prv) is the ultimate phosphoryl donor. A second major function of the PTS is to regulate the utilization of certain non-PTS substrates such as glycerol, maltose, and melibiose in *Salmonella typhimurium* and also lactose in *Escherichia coli*. In media containing both a PTS and a non-PTS sugar, the PTS sugar is utilized before induction of the catabolic systems for the non-PTS sugar (diauxic growth, "the glucose effect"). This form of PTS-mediated repression involves both the inhibition of uptake of the non-PTS sugar (inducer exclusion) and regulation of adenylate cyclase. We have previously reported (2) that a single mutation, designated *crr*, can overcome PTS-mediated repression; the *crr* locus maps next to (3), but is not part of, the *pts* operon [which includes the structural genes for enzyme I and the histidine-containing phosphocarrier protein of the PTS (HisPr; normally designated HPr)]. In earlier studies (2), *crr* mutants were shown to contain normal levels of PTS proteins, as measured by their phosphotransferase activities *in vitro*, with the exception of one protein—the phosphocarrier protein of the PTS specific for glucose or methyl α-glucoside (III^{Glc}). The *crr* mutants contained low activities of III^{Glc}. However, these results could not distinguish between two possibilities: (*i*) that *crr* is the structural gene for III^{Glc}, so that mutants would be expected to contain normal levels of altered III^{Glc}, or (*ii*) that *crr* regulates the synthesis of III^{Glc}, so that the mutants would be expected to contain abnormal levels of the normal protein.

This report presents two independent lines of evidence indicating that *crr* is the structural gene for III^{Glc}. First, pure III^{Glc} isolated from a crr^- mutant strain of *S. typhimurium* exhibited only 2–3% of the activity of wild-type III^{Glc} in the *in vitro* sugar phosphorylation assay. Second, a small fragment [1.3 kilobase (kb)] cloned from *E. coli* was shown to transform crr^- to crr^+ cells and to direct the production of III^{Glc}. The structural gene for III^{Glc} requires a minimum of 0.6 kb pairs.

MATERIALS AND METHODS

Bacterial Strains and Growth. *S. typhimurium* and *E. coli* strains used in these studies are listed in Table 1. *E. coli* strain DS166 was constructed by P-1 transduction into DG40 of the *recA* mutation of JC10240 selecting for tetracycline resistance and screening for UV sensitivity. Selection for loss of Tn*10* was accomplished by the method of Bochner *et al.* (7) as modified by Maloy and Nunn (8), after which cells were again tested for the presence of the *recA* mutation by measuring UV sensitivity. Cells were grown in media as described (9–11).

The presence of a functional crr^+ was determined by examining the cells for their ability to ferment maltose in the presence of methyl α-glucoside (αMeGlc) on MacConkey agar indicator plates (without lactose) (2, 3, 12) containing 2% maltose, 10 mM αMeGlc, and—where appropriate—tetracycline at 10 μg/ml. Cells defective in enzyme I activity ($ptsI^-$) form white nonfermenting colonies, whereas $ptsI^-$ crr^- cells in which PTS-mediated repression is relieved form dark red fermenting colonies.

Cells were transformed with plasmids by using a modification of the $CaCl_2$ method of Mandel and Higa (13).

Assays for III^{Glc}. In the *in vitro* reactions catalyzed by the PTS, when αMeGlc is the sugar substrate, the phosphoryl group from *P-e*Prv is transferred sequentially to enzyme I, HisPr, III^{Glc}, and finally to αMeGlc, the last step catalyzed by the integral membrane protein of the PTS specific for glucose

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: PTS, phospho*enol*pyruvate:glycose phosphotransferase system; *P-e*Prv, phospho*enol*pyruvate; HisPr, histidine-containing phosphocarrier protein of the phosphotransferase system; III^{Glc}, the phosphocarrier protein of the phosphotransferase system specific for glucose or methyl α-glucoside; III^{Glc}_{slow}, the form of III^{Glc} with low electrophoretic mobility; III^{Glc}_{fast}, the form of III^{Glc} with high electrophoretic mobility; II-B^{Glc}, the integral membrane protein of the phosphotransferase system specific for glucose or methyl α-glucoside; αMeGlc (or methyl α-glucoside), methyl α-D-glucopyranoside; αMeGlc-*P*, methyl α-D-glucopyranoside 6-phosphate; kb, kilobase.

or αMeGlc (II-B^{Glc}). When II-B^{Glc} is made rate-limiting (14), phospho-III^{Glc} and αMeGlc are cosubstrates of this enzyme. Under conditions in which αMeGlc is used at "substrate-saturating" levels and enzyme I and HisPr are in large excess, typical Michaelis–Menten-type kinetics are observed with homogeneous III^{Glc} (11), and from these kinetics it is possible to calculate apparent K_m and V_{max} values. In these assays, the rate of αMeGlc phosphorylation is followed as a function of incubation time by the ion-exchange column procedure (14).

A spectrophotometric assay (14) was used to measure the rate and extent of phosphotransfer from *P-e*Prv to III^{Glc} (in the presence of enzyme I and HisPr).

The preparation of homogeneous *S. typhimurium* enzyme I and HisPr is described elsewhere (9, 10) as is the partial purification of II-B^{Glc} (14), the preparation used in this study.

It is important to emphasize that unlike the PTS proteins of *Staphylococcus aureus*—which are unable to significantly substitute for the corresponding proteins of *E. coli* in the *in vitro* assay (15)—the proteins from *S. typhimurium* and *E. coli* apparently crossreact at 100% efficiency. In fact, about 75% of *E. coli* HisPr has been subjected to sequence analysis and is identical to *S. typhimurium* HisPr, which has been completely analyzed (16).

The quantity of III^{Glc}, both mutant and wild-type proteins, was determined by modifications (11) of the rocket immunoelectrophoresis technique (17, 18). Anti-III^{Glc} serum was obtained (11) by using homogeneous *S. typhimurium* III^{Glc}_{slow} (see below) and was found to crossreact with *E. coli* III^{Glc}, as expected.

Protein was measured by the method of Bradford (19), calibrated with pure III^{Glc} (11) as standard.

Purification of III^{Glc}. In the results described elsewhere (11), two methods were used for isolating homogeneous III^{Glc} from *S. typhimurium* LT-2. Procedure A involved classical fractionation techniques. With the availability of the pure protein, antibodies were obtained and coupled to agarose, and affinity chromatography was used as the first step in procedure B (giving about 50% pure protein in one step). In both procedures, two electrophoretically distinguishable forms of III^{Glc} were isolated. The more rapidly migrating species, III^{Glc}_{fast}, was shown to have about 5% of the activity of the slower species, III^{Glc}_{slow}, in the sugar phosphorylation assay *in vitro*. III^{Glc}_{slow} was shown to contain an NH_2-terminal heptapeptide missing in III^{Glc}_{fast}.

In the present studies, III^{Glc} was purified from the *crr*$^-$ mutant, *S. typhimurium* SB1796, by using immunoelectrophoresis as the method for assaying the protein during purification. Procedure B (11) was slightly modified for this purpose by omission of the second step. In brief, crude extracts of SB1796 (grown on nutrient broth) were passed over the antibody column, and the column was then washed with 25 mM Tris·HCl buffer (pH 7.9) containing 1 mM EDTA and 0.15 M NaCl. III^{Glc} was eluted from the column with 0.5 M acetic acid and immediately was neutralized with Tris base and concentrated using a Pellicon PTGA membrane (Millipore). The fraction was then dialyzed against 200 mM Hepes/82.5 mM Tris buffer, pH 7.5, and subjected to preparative polyacrylamide gel electrophoresis as described for wild-type III^{Glc} (11). This method separates III^{Glc}_{slow} and III^{Glc}_{fast}. The relevant fractions were combined, concentrated as described above, dialyzed against 25 mM Tris·HCl buffer (pH 7.5) containing 1 mM EDTA, and stored at −20°C.

$NaDodSO_4$/polyacrylamide gel electrophoresis was performed by the method of Laemmli (20) by using 1.5-mm thick gels in an apparatus manufactured by Bio-Rad.

Maxicell Labeling of Proteins. Proteins encoded by plasmids pBR322 and pDS45 were examined by the maxicell method as described by Sancar *et al.* (21). Overnight cultures grown on Luria broth (10 g of Bactotryptone, 5 g of yeast extract, and 5 g of NaCl per liter) plus tetracycline at 10 μg/ml were started from single colonies isolated from MacConkey agar plates containing 2% maltose, 10 mM αMeGlc, and tetracycline at 10 μg/ml. These cultures were used to inoculate cultures of medium (20 ml) with the following composition (per liter): 1% casamino acids; thiamine HCl, 2 μg/ml; 0.1% glucose; tetracycline, 10 μg/ml; $Na_2HPO_4 \cdot 7\ H_2O$, 10 g; KH_2PO_4, 3 g; NaCl, 0.5 g; NH_4Cl, 1 g; $MgSO_4$, 25 mg; $CaCl_2$, 4 mg. Aliquots (10 ml) of cultures in midexponential growth phase were added to glass Petri dishes (inside diameter = 6 cm) and irradiated with a Westinghouse Sterilamp at a distance of 800 cm. CRS603 strains were irradiated for 2–10 sec and DS166 strains for 60 sec while the cultures were gently swirled. UV-irradiated cultures were then shaken at 37°C for 1 hr in 125-ml Erlenmeyer flasks, after which time cycloserine was added to a final concentration of 100 μg/ml. After incubation for 10–15 hr at 37°C, cells were washed twice with sulfate-free Hershey salts (22) and resuspended in 2.5 ml of sulfate-free Hershey medium. The cells were then incubated for 1 hr at 37°C with reciprocal shaking in 50-ml flasks after which time [^{35}S]methionine (Amersham, >1,290 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was added to a final concentration of 5 μCi/ml. Cells were incubated with shaking for another 60–90 min and were then centrifuged at 3,000 × *g* for

Table 1. Bacterial strains

Strain	Relevant genotype	Parent	Defect	Source/reference
S. typhimurium				
LT-2	Wild type		None	(4)
SB1475	*ptsH15*	LT-2	HisPr	(4)
SB1476	*ptsI17*	LT-2	Enzyme I	(4)
SB1477	*ptsI18*	LT-2	Enzyme I	(4)
SB1687	*manA12*	LT-2	Enzyme II^{Man}	(4)
SB1796	*ptsI17crr-1*	*ptsI17*	Enzyme I, crr	(2)
SB1798	*ptsI18crr-3*	*ptsI18*	Enzyme I, crr	(2)
SB1799	*ptsI19crr-4*	*ptsI19*	Enzyme I, crr	(2)
SB2026	*ptsH15crr-6*	*ptsA15*	HisPr, crr	(2)
E. coli				
DG2	Hfr K16 *thi ptsI*::λ $cI_{857}red_3$			W. Epstein*
DG40	Hfr K16 *thi relA1* (*ptsI-cysA*)			W. Epstein; CGSC 5915†
JC10240	Hfr P045 *srlC300*::Tn*10 recA56*			(5)
DS166	DG40 *recA56*			This work
CRS603	*recA1 uvrA6 phr-1*			(6); CGSC 5830

* Dept. of Biochemistry, Univ. Chicago, Chicago, IL 60637.
† CGSC *E. coli* Genetic Stock Center, Dept. of Human Genetics, Yale Univ. School of Medicine, New Haven, CT 06510 (B. J. Bachman, Curator).

10 min at 4°C. Cell pellets were resuspended in 120 μl of $NaDodSO_4$ lysis buffer (62.5 mM Tris·HCl, pH 6.0/10% glycerol/2% $NaDodSO_4$/0.001% bromphenol blue) and were boiled for 2 min before being analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. Samples to be analyzed by rocket immunoelectrophoresis were resuspended in 81 mM Tris/24 mM Tricine buffer, pH 8.8/1% Triton X-100 and lysed by sonication in a bath sonicator (five 15-sec bursts with cooling on ice between bursts), followed by three rounds of freezing and thawing.

RESULTS

Isolation and Properties of III^{Glc} from *crr*⁻ Mutant. Initial screening experiments of crude extracts by rocket immunoelectrophoresis showed that one *S. typhimurium crr*⁻ mutant—strain SB2026—contained no detectable III^{Glc}, but that three mutants—SB1796, SB1798, and SB1799—each contained approximately normal levels (60–100% of wild type) of protein that crossreacted with anti-III^{Glc} serum. Because the same extracts exhibited low levels of III^{Glc} activity in the phosphorylation of αMeGlc (2), these results implied that the mutants contained altered III^{Glc} proteins that were still antigenic.

This conclusion was further substantiated as follows. One of the steps in procedure A (11) involves Sephadex G-75 chromatography, which separates wild-type III^{Glc} from most of the protein in a crude fraction. The chromatography was performed at 4°C with the column equilibrated and developed with 25 mM Tris·HCl buffer, pH 7.9/1 mM EDTA/50 mM KCl. Under these conditions, the mutant proteins gave two peaks, one in the expected position for III^{Glc}, but most of the mutant III^{Glc} eluted with the bulk protein. This altered behavior was subsequently found also with partially purified (50% homogeneous) protein from SB1796, SB1798, and SB1799. These results were obtained not simply because the mutant III^{Glc} was of higher molecular weight, as shown by the following results. When Sephadex G-75 chromatography was performed at room temperature rather than at 4°C and the column was equilibrated and developed with 40 mM Hepes/26 mM Tris, pH 7.5, with 5% (vol/vol) glycerol, the mutant proteins behaved identically to III^{Glc} from strains LT-2, SB1476, and SB1477; the antibody-reactive proteins from mutants eluted as symmetrical single peaks and at the position expected for a M_r 20,000 protein.

Procedure B was used to isolate each species, III^{Glc}_{slow} and III^{Glc}_{fast}, from the mutant strain, SB1796, in an overall yield of about 30%, of which ≈90% was III^{Glc}_{slow}. The purity of III^{Glc}_{slow} from LT-2 and SB1796 is shown by the $NaDodSO_4$/polyacrylamide electrophoresis gels in Fig. 1, lanes 2 and 3. Because 10 μg of protein was used in these gels and no other bands were visualized, the proteins were concluded to be >99% homogeneous.

III^{Glc}_{slow} is converted to III^{Glc}_{fast} by a membrane-bound protease that is apparently specific for this reaction. Because the mutant SB1796 also contains both species, III^{Glc}_{slow} and III^{Glc}_{fast}, and in about the same ratio as the wild type ($III^{Glc}_{slow} > III^{Glc}_{fast}$) (Fig. 1, lane 1), the protease is present and active in the *crr*⁻ strain.

Both III^{Glc}_{slow} and III^{Glc}_{fast} from SB1796 exhibit M_r ≈20,000 in $NaDodSO_4$/polyacrylamide gel electrophoresis and are electrophoretically identical to the respective III^{Glc} proteins from LT-2 when examined by native polyacrylamide gel electrophoresis in the presence of glycerol. The mutant III^{Glc} proteins are also indistinguishable from the wild-type species by amino acid analysis, by immunological criteria, and by peptide mapping after trypsin treatment.

However, the mutant proteins do show different physical properties from the wild-type III^{Glc} species in the absence of glycerol. Their anomalous behavior on gel filtration is described above. This change was also evident in polyacrylamide gel electrophoresis under nondenaturing conditions. Whereas Fig. 1 shows that the mutant forms of III^{Glc}_{slow} and III^{Glc}_{fast} migrated as single protein bands with the same mobilities as the wild-type proteins, these results were obtained only when the stacking and resolving gels contained 5% glycerol. Omission of glycerol from the gels had little or no effect on the wild-type proteins. In sharp contrast, the mutant proteins showed completely altered behavior in the absence of glycerol. Variable results were obtained, apparently depending on the length of storage of the protein (−20°C) and how many times the sample was frozen and thawed. In some cases multiple bands were observed, including a band at the origin of the resolving gel—a small quantity of which migrated at the same rate as the wild-type protein—and several bands in between these extremes. In other experiments with the same preparation, only a band at the origin was evident, whereas in yet others, a diffuse, slowly migrating protein band was detected.

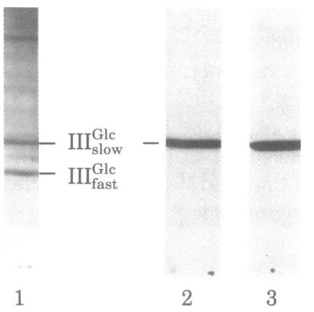

FIG. 1. Analytical polyacrylamide gel electrophoresis and $NaDodSO_4$/polyacrylamide electrophoresis gels of III^{Glc} from *S. typhimurium* strains LT-2 and SB1796. Lane 1, native polyacrylamide gel electrophoresis (3495.8, ref. 11) of partially purified SB1796 III^{Glc} (procedure B, step 1); 6 μg of protein. This gel was stained with the silver stain of Wray *et al.* (23). Lanes 2 and 3, $NaDodSO_4$/polyacrylamide gel electrophoresis (20) of homogeneous III^{Glc}_{slow} from LT-2 (lane 2, 10 μg) and SB1796 (lane 3, 10 μg). These gels were stained with Coomassie brilliant blue R.

We emphasize that aliquots of the same solutions showed normal behavior in nondenaturing gels containing 5% glycerol and in $NaDodSO_4$/polyacrylamide gel electrophoresis. These, as well as the Sephadex gel filtration results, indicate that the mutant proteins—unlike the wild-type proteins—aggregate in the absence of glycerol.

Phosphoacceptor and Phosphotransfer Activities of III^{Glc}_{slow} from SB1796. The spectrophotometric assay was used to measure the ability of III^{Glc}_{slow} from SB1796 to accept a phosphoryl group from phospho-HisPr. Again the mutant protein behaved identically to III^{Glc}_{slow} from strain LT-2. The rates of phosphorylation were indistinguishable, and both proteins accepted one mole of phosphate per mole of protein.

However, a clear difference between mutant and wild-type III^{Glc}_{slow} proteins was evident in measuring the phosphotransfer ability of the two proteins in the complete PTS assay—i.e., the phosphorylation of αMeGlc. The results are shown in Fig. 2. III^{Glc}_{slow} from the mutant shows ≈2% of the activity obtained with III^{Glc}_{slow} from LT-2.

Experiments in which SB1796 III^{Glc}_{slow} was mixed with LT-2 III^{Glc}_{slow} showed no inhibition of the activity of the latter protein. Thus, the low activity of SB1796 III^{Glc}_{slow} is not caused by the presence of an inhibitor of sugar phosphorylation.

In sum, these results show that the *crr*⁻ mutant contains normal levels of an abnormal III^{Glc} protein.

Cloning the *E. coli crr* Gene. A specialized λ transducing phage, λ*pcysAcrr*, carrying a functional *crr* gene was isolated by inducing the *E. coli* lysogen DG2 that contains $\lambda cI_{857}red_3$ inserted at a secondary *att* site within the *ptsI* gene adjacent to

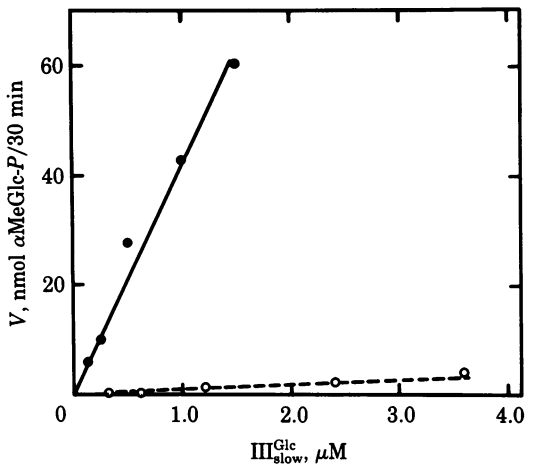

FIG. 2. The relative activity of III$^{Glc}_{slow}$ from *S. typhimurium* strains LT-2 and SB1796. Sugar phosphorylation assays *in vitro* were performed as described (11, 14) by using partially purified enzyme II-B^{Glc} (14). Each incubation mixture contained 50 mM Tris·HCl buffer (pH 8.0); 10 mM *P-ePrv*; 12.5 mM potassium fluoride; 5 mM magnesium chloride; 0.2 mM dithioerythritol; 7 mM ^{14}C-labeled αMeGlc, 1.4×10^5 cpm/μmol; 4 units of enzyme I [step 5 (9)]; 3.5 μM HisPr [step 4 (10)]; 0.35 unit of enzyme II-B^{Glc} (14) (total volume, 100 μl). The mixtures were incubated for 30 min at 37°C and the ^{14}C-labeled αMeGlc-*P* was measured by the ion exchange method (14). ●, LT-2 III$^{Glc}_{slow}$; ○, SB1796 III$^{Glc}_{slow}$.

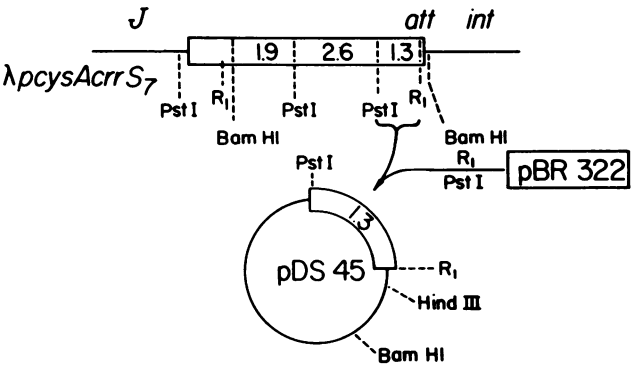

FIG. 3. Restriction endonuclease maps of transducing phage of plasmid carrying the *crr* gene. The linear map in the upper part of the figure shows a portion of the specialized transducing phage λ*pcysAcrrS*$_7$. The thin line represents the phage genome and includes the *J*, *att*, and *int* genes of λ. The rectangle located between *J* and *att* consists of *E. coli* genomic DNA and contains *cysA* and *crr*. Restriction endonuclease cleavage sites are indicated by the dashed lines, and the numbers in the rectangle give the length of DNA obtained between the cleavage points as kb pairs, measured by agarose gel electrophoresis (25). The fragments obtained after treating the λ*pcysAcrrS*$_7$ DNA with the indicated enzymes were cloned into pBR322. The 1.3-kb fragment yielded the plasmid pDS45 and, as indicated in the text, carried a functional *crr* gene. This plasmid also contains a functional tetracycline resistance gene but does not make β-lactamase; transformed cells harboring the plasmid are therefore resistant to tetracycline but not to ampicillin. R$_1$, *Eco*RI.

crr gene (24). The S_7 mutation that prevents lysis of the host cell by the phage was subsequently crossed into λ*pcysAcrr* to yield λ*pcysAcrrS*$_7$. The presence of a functional *crr* gene in the phage was demonstrated by the fact that *ptsI*$^-$ *crr*$^-$ strains that are able to ferment maltose in the presence of 10 mM αMeGlc lose this capacity upon becoming lysogenized with λ*pcysAcrrS*$_7$.

In addition to causing the *crr*$^-$ phenotype to revert to *crr*$^+$, λ*pcysAcrrS*$_7$ directs the synthesis of III^{Glc}. *E. coli* DG40 is a strain deleted for *crr* and produces no III^{Glc}. However, λ*pcysAcrrS*$_7$ lysogens of DG40 produce III^{Glc} as shown by rocket immunoelectrophoresis (data not shown).

The phage λ*pcysAcrrS*$_7$ is λ*gal*-like in structure with about 8.5 kb of bacterial DNA replacing DNA between the attachment site and the *J* gene. Two *Eco*RI, two *Pst* I, and one *Bam*HI restriction sites were mapped in the bacterial DNA segment (Fig. 3) and various fragments generated by digestion with these enzymes were subcloned into the plasmid pBR322. In particular, one plasmid, pDS45 (Fig. 3)—carrying a 1.3-kb fragment from the transducing phage—was found to carry a functional *crr* gene. All *ptsI*$^-$ *crr*$^-$ strains that were transformed with pDS45 showed restoration of PTS-mediated repression of utilization of maltose, lactose, glycerol, and melibiose. Cell extracts of DG40 harboring pDS45 were shown to contain III^{Glc} by rocket immunoelectrophoresis (see below).

Maxicell Labeling of Proteins. To provide evidence that the *crr* gene carried on pDS45 is the structural gene for III^{Glc}, we examined the synthesis of proteins directed by pDS45 using the maxicell method of Sancar *et al.* (21). When RecA$^-$ cells harboring a high-copy-number recombinant plasmid are treated with a suitable dose of UV radiation, the host DNA is damaged and eventually degraded, whereas some plasmids escape damage because of the large number of copies present in the cell. When [^{35}S]methionine is added to these treated cells, the synthesis of proteins encoded by the host DNA is found to be dramatically decreased, whereas proteins encoded by genes on the plasmid continue to be synthesized and are readily labeled. Thus, if the plasmid pDS45 carries the structural gene for III^{Glc}, label should be specifically incorporated into III^{Glc}.

Fig. 4*B* shows that pDS45 directs the synthesis of only a single protein containing [^{35}S]methionine (lane 2). This protein comigrates with III^{Glc} (lane 4). [III^{Glc} is known to contain three methionines by amino acid analysis (11).] The plasmid pDS45 does not direct the synthesis of β-lactamase, which is consistent with the fact that the fragment carrying the *crr* gene is cloned between the *Pst* I and *Eco*RI sites of pBR322, replacing the promoter and the NH_2-terminal segment (26) of β-lactamase. Identical results were obtained when ^{14}C-labeled amino acids were used in place of [^{35}S]methionine.

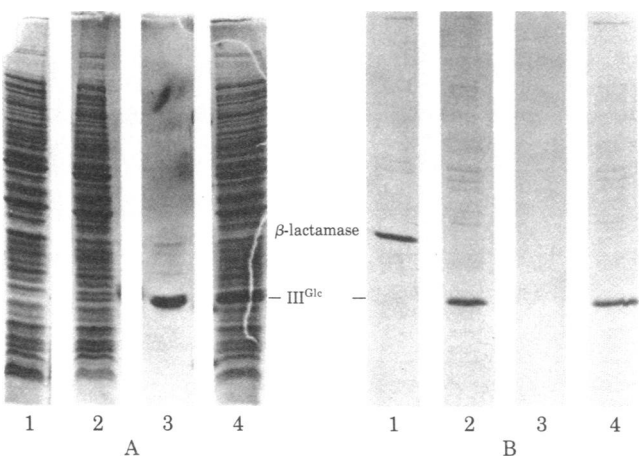

FIG. 4. NaDodSO$_4$/polyacrylamide gel electrophoresis of crude extracts of [^{35}S]methionine-labeled CRS603 cells. The cells harboring the plasmids were subjected to UV irradiation and labeled, and crude extracts were prepared as described. (*A*) Aliquots of the extracts were subjected to NaDodSO$_4$/polyacrylamide gel electrophoresis and the gels were stained with Coomassie blue. (*B*) Autoradiographs of the stained gels. Other aliquots of the extracts were treated with 10% trichloroacetic acid, and the trichloroacetic acid-precipitable cpm were determined. The following proteins were electrophoresed: lane 1, crude extract of CRS603 (pBR322), 71,000 trichloroacetic acid-precipitable cpm; lane 2, crude extract of CRS603 (pDS45), 122,000 trichloroacetic acid-precipitable cpm; lane 3, 10 μg of pure III$^{Glc}_{slow}$; lane 4, same as lane 2 plus 10 μg of III$^{Glc}_{slow}$.

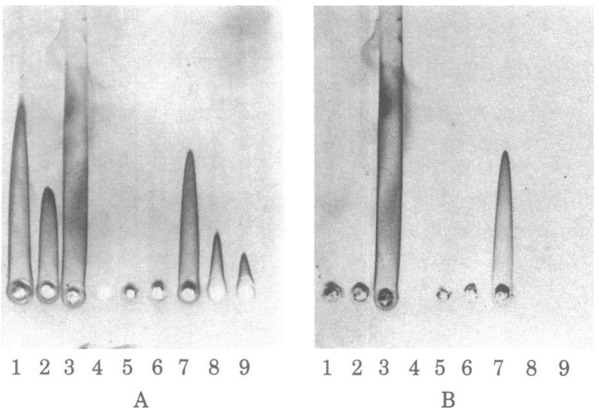

FIG. 5. Rocket immunoelectrophoresis of crude extracts of labeled *E. coli* maxicells. Cells with and without plasmids were exposed to UV light and labeled with [^{35}S]methionine, and crude extracts were prepared in 1% Triton X-100 as described. Aliquots of the extracts (18 μl each) were examined by rocket immunoelectrophoresis (11, 17, 18). The gel was stained with Coomassie blue (*A*), followed by autoradiography (*B*). The wells contained the following components (crude extracts unless otherwise noted): 1, CRS603; 2, CRS603 (pBR322); 3, CRS603 (pDS45); 4, no sample; 5, DS166; 6, DS166 (pBR322); 7, DS166 (pDS45); 8, 56 ng of III^{Glc}_{slow}; 9, 28 ng of III^{Glc}_{slow}. CRS603 is a *crr*$^+$ strain, whereas DS166 is deleted for the *crr* gene.

$NaDodSO_4$/polyacrylamide gel electrophoresis of crude extracts of maxicells does not resolve III^{Glc}_{fast} and III^{Glc}_{slow} sufficiently to unequivocally identify III^{Glc}_{fast} in the crude extracts (11). However, cells harboring the plasmids containing the *crr* genes produce both species of the proteins (due to host protease).

Rocket Immunoelectrophoresis. Rocket immunoelectrophoresis was used to show that the protein that comigrates with III^{Glc} on $NaDodSO_4$/polyacrylamide electrophoresis gels described above is immunologically related to III^{Glc}. Fig. 5 shows III^{Glc} rockets with extracts of cells labeled by the maxicell technique. CRS603 strains all contain endogenous III^{Glc} as shown by the presence of Coomassie blue-stained rockets in lanes 1 and 2 of Fig. 5*A*. However, only CRS603 harboring pDS45 gives a radioactive rocket (Fig. 5*A*, lane 3; Fig. 5*B*, lane 3), indicating that the synthesis of III^{Glc} is being directed by the plasmid. *E. coli* strain DS166 is deleted for *crr* and produces no III^{Glc} rockets (lane 5), as expected, and the same results are obtained with DS166 (pBR322) (lane 6), as expected. By contrast, DS166 that harbors pDS45 produces a III^{Glc} rocket that is detected both by Coomassie blue-staining and autoradiography (Fig. 5*A*, lane 7; Fig. 5*B*, lane 7). Thus, the plasmid pDS45 directs the synthesis of III^{Glc}.

DISCUSSION

The preferential utilization of a PTS sugar in the presence of a non-PTS sugar, commonly called diauxie, is a form of PTS-mediated repression—in this case, repression of induction of synthesis of the systems required for catabolism of the non-PTS sugar. PTS-mediated repression has been explored in detail in earlier (2) and more recent (27) studies from this laboratory, particularly with respect to PTS regulation of the systems required for transport of the following non-PTS compounds: glycerol, maltose, melibiose, and lactose. These transport systems, even when fully induced, are inhibited by PTS sugars, including nonmetabolizable analogues such as methyl α-glucoside; leaky enzyme I (or HisPr) mutants are hypersensitive to this inhibition. The inhibition occurs directly at the level of the functional permeases (27), not at the levels of either the generation of metabolic energy or coupling of energy to the transport system.

These diverse and profound physiological effects of the PTS are completely reversed by a single mutation in a gene designated *crr*. The present studies provide evidence that *crr* is the structural gene for the PTS protein III^{Glc}. As part of these studies, the *crr* gene has been cloned on a small (1.3 kb) fragment of DNA. This result has important theoretical and practical implications. For instance, it should now be possible to determine whether III^{Glc}_{slow} is a primary translation product of *crr* or has been processed after translation, as is true of III^{Glc}_{fast}. In addition, the physiological behavior of cells containing high (10-fold over normal) levels of III^{Glc} is of considerable interest, and preliminary experiments have shown that such cells are unable to utilize a wide variety of compounds.

Identification of III^{Glc} as the *crr* gene product is, we believe, of great importance. (*i*) Biochemical experiments can now be performed to determine precisely how the PTS regulates adenylate cyclase and the non-PTS transport systems. For instance, our earlier model (28) of direct inhibition of non-PTS permeases by III^{Glc} but not by phospho-III^{Glc} can be tested. (*ii*) The roles of III^{Glc}_{slow}, III^{Glc}_{fast}, and the apparently specific membrane-bound protease that converts III^{Glc}_{slow} to III^{Glc}_{fast} can be determined. (*iii*) This direct link between PTS and non-PTS transport systems may permit further exploration of the idea (1) that the non-PTS systems have evolved by modification of the PTS.

We thank Dr. Wolfgang Epstein and Dr. Mark Pearson for gifts of *E. coli* strains and Dr. W. Dean Rupp for helpful advice concerning the maxicell method. This study was supported by a grant from the National Institutes of Health (21901). This is contribution 1136 from the McCollum–Pratt Institute.

1. Postma, P. W. & Roseman, S. (1976) *Biochim. Biophys. Acta* **457**, 213–257.
2. Saier, M. H., Jr., & Roseman, S. (1976) *J. Biol. Chem.* **251**, 6598–6605.
3. Cordaro, J. C. & Roseman, S. (1972) *J. Bacteriol.* **112**, 17–29.
4. Saier, M. H., Jr., Simoni, R. D. & Roseman, S. (1976) *J. Biol. Chem.* **251**, 6584–6597.
5. Csonka, L. N. & Clark, A. J. (1980) *J. Bacteriol.* **143**, 529–530.
6. Sancar, A. & Rupert, C. S. (1978) *Nature (London)* **272**, 471–472.
7. Bochner, B. R., Huang, H., Schieven, G. L. & Ames, B. N. (1980) *J. Bacteriol.* **143**, 926–933.
8. Maloy, S. R. & Nunn, W. D. (1981) *J. Bacteriol.* **145**, 1110–1112.
9. Weigel, N., Waygood, E. B., Kukuruzinska, M. A., Nakazawa, A. & Roseman, S. (1982) *J. Biol. Chem.*, in press.
10. Beneski, D. A., Nakazawa, A., Weigel, N., Hartman, P. E. & Roseman, S. (1982) *J. Biol. Chem.*, in press.
11. Meadow, N. D. & Roseman, S. (1982) *J. Biol. Chem.*, in press.
12. Difco Laboratories (1953) *Difco Manual* (Difco Laboratories, Detroit, MI), 9th Ed., pp. 131–132.
13. Mandel, H. & Higa, A. (1970) *J. Mol. Biol.* **53**, 159–162.
14. Waygood, E. B., Meadow, N. D. & Roseman, S. (1979) *Anal. Biochem.* **95**, 293–304.
15. Simoni, R. D., Nakazawa, A., Hays, J. B. & Roseman, S. (1973) *J. Biol. Chem.* **248**, 932–940.
16. Weigel, N., Powers, D. A. & Roseman, S. (1982) *J. Biol. Chem.*, in press.
17. Laurell, C.-B. (1972) *Scand. J. Clin. Lab. Invest.* **29**, Suppl. 124, 21–37.
18. Weeke, B. (1973) *Scand. J. Immunol.* **2**, Suppl. 1, 37–46.
19. Bradford, H. H. (1976) *Anal. Biochem.* **72**, 248–254.
20. Laemmli, U. K. & Ravre, M. (1973) *J. Mol. Biol.* **80**, 575–599.
21. Sancar, A., Wharton, R. P., Seltzer, S., Kacinski, B. M., Clarke, N. D. & Rupp, W. D. (1981) *J. Mol. Biol.* **148**, 45–62.
22. Worcel, A. & Burgi, E. (1974) *J. Mol. Biol.* **82**, 91–105.
23. Wray, W., Boulikas, T., Wray, V. P. & Hancock, R. (1981) *Anal. Biochem.* **118**, 197–203.
24. Gottesman, H. H. (1976) *Virology* **72**, 33–44.
25. Sharp, P. A., Snyder, B. & Sambrook, J. (1973) *Biochem.* **12**, 3055–3063.
26. Sutcliffe, J. G. (1979) *Cold Spring Harbor Symp. Quant. Biol.* **43**, 77–90.
27. Mitchell, W. J., Misko, T. P. & Roseman, S. (1982) *J. Biol. Chem.*, in press.
28. Roseman, S. (1977) in *Biochemistry of Membrane Transport*, FEBS Symposium 42, eds. Semenza, G. & Carafoli, E. (Springer, Berlin), pp. 582–597.