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Characterization of Factor III^{Glc} in Catabolite Repression-Resistant (*crr*) Mutants of *Salmonella typhimurium*

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crr mutants of *Salmonella typhimurium* are thought to be defective in the regulation of adenylate cyclase and a number of transport systems by the phosphoenolpyruvate-dependent sugar phosphotransferase system. *crr* mutants are also defective in the enzymatic activity of factor III^{Glc} (III^{Glc}), a protein component of the phosphotransferase system involved in glucose transport. Therefore, it has been proposed that III^{Glc} is the primary effector of phosphotransferase system-mediated regulation of cell metabolism. We characterized *crr* mutants with respect to the presence and function of III^{Glc} by using an immunochemical approach. All of the *crr* mutants tested had low (0 to 30%) levels of III^{Glc} compared with wild-type cells, as determined by rocket immunoelectrophoresis. The III^{Glc} isolated from one *crr* mutant was investigated in more detail and showed abnormal aggregation behavior, which indicated a structural change in the protein. These results supported the hypothesis that a *crr* mutation directly affects III^{Glc}, probably by altering the structural gene of III^{Glc}. Several *crr* strains which appeared to be devoid of III^{Glc} in immunoprecipitation assays were still capable of in vitro phosphorylation and transport of methyl α-glucoside. This phosphorylation activity was sensitive to specific anti-III^{Glc} serum. Moreover, the membranes of *crr* mutants, as well as those of wild-type cells, contained a protein that reacted strongly with our anti-III^{Glc} serum. We propose that *S. typhimurium* contains a membrane-bound form of III^{Glc} which may be involved in phosphotransferase system activity.

The phosphoenolpyruvate-dependent sugar phosphotransferase systems (PTS) of *Escherichia coli* and *Salmonella typhimurium* are involved both in sugar transport and in regulation of cell metabolism (4, 9, 23) (Fig. 1). A manifestation of this regulatory function is the pleiotropic nature of *ptsH,I* mutants, which are deficient in one or both of the general PTS components enzyme I and HPr. *pts* mutants are not able to grow on a large number of substrates which are not transported by the PTS (23). A common feature of these substrates is that the genes involved in their catabolism are all subject to catabolite repression; i.e., they depend upon the presence of cyclic AMP and the cyclic AMP binding protein. Generally, expression of the genes which are repressed by a *ptsH,I* mutation also depends upon the presence of an inducer.

The growth defects of *ptsH,I* mutants have been connected with two experimental phenomena which suggest an important regulatory function of the PTS. First, the transport of glycerol, maltose, and melibose, which are not substrates of the PTS, is inhibited by PTS substrates. Neither direct competition nor metabolism of the PTS substrates can account for this phenomenon, which is known as inducer exclusion (29, 30). This inhibition can be explained by a model in which variations in the phosphorylation state of the PTS directly affect the activity of unrelated transport systems (23, 27, 32). The second phenomenon is that the activity of adenylate cyclase appears to be affected by changes in the phosphorylation state of the PTS (5, 8, 21). It is thought that PTS-mediated regulation of both adenylate cyclase and solute transport is responsible for the pleiotropic character of *ptsH,I* mutants. The inability to phosphorylate a hypothetical regulator protein would result in a permanently repressed state of *ptsH,I* mutants (23). Therefore, characterization of pseudorevertants of *ptsHI* deletion mutants which regain the capacity to grow on one or more of the non-PTS substrates should provide information about the mechanism of PTS-mediated regulation of cell metabolism. We studied one class of such mutants of *S. typhimurium* (*crr* mutants). A mutation in the gene designated *crr* permits growth of a Δ(*ptsHI*) strain on melibiose, glycerol, and maltose; such a mutation was described originally by Saier and Roseman (23, 28, 29). The *crr* gene is situated near the *pts* operon on the

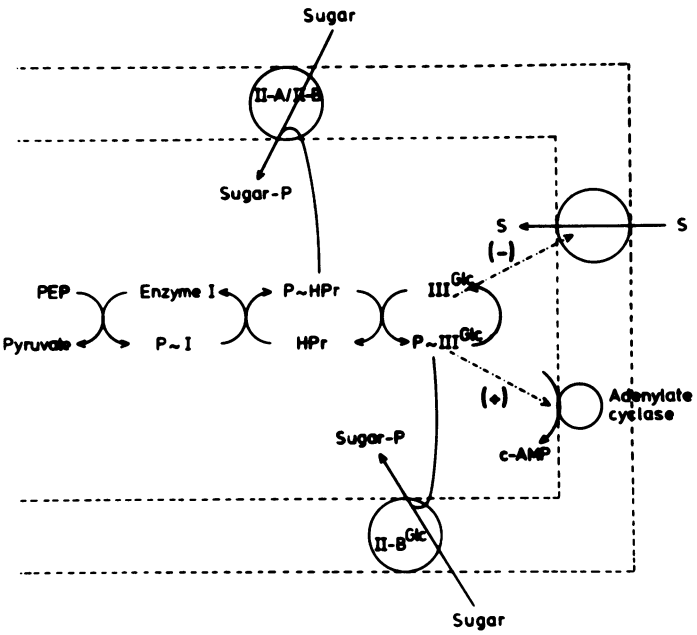

FIG. 1. Schematic representation of the PTS. The PTS consists of two general proteins (enzyme I and HPr) and a number of sugar-specific proteins. This figure shows the II-A/II-B and III^{Glc}/II-B^{Glc}systems, which are able to transport mannose, glucose, and 2-deoxyglucose and glucose and methyl α-glucoside, respectively. PEP, phosphoenolpyruvate; Sugar-P, sugar phosphate.

genetic map (2). A *crr* mutation can abolish the inhibition of transport via unrelated transport systems by the PTS (29) and can also affect the regulation of adenylate cyclase (5). In fact, *crr* mutants of *S. typhimurium* are partially defective in adenylate cyclase activity, which explains their characteristic phenotype (5, 31).

Another characteristic of *crr* mutants is a defect in methyl α-glucoside phosphorylation and transport. This defect is caused by a lowered enzymatic activity of factor III^{Glc} (III^{Glc}) (2, 11, 24, 28), which is a protein component of the PTS that is involved in the transport of glucose via enzyme II-B^{Glc} (23) (Fig. 1). This finding has led to the hypothesis that III^{Glc} is either the primary effector of PTS-mediated regulation or at least closely associated with such a regulator protein (23, 29). However, no direct evidence for the role of III^{Glc} in the regulation of adenylate cyclase activity and transport has been presented until now. The function of the *crr* gene, in particular with respect to the production of III^{Glc}, has not been explained fully. It is not clear whether *crr* is the structural gene of III^{Glc} or whether mutations in the *crr* gene affect III^{Glc} function in an indirect way. To investigate this further, we isolated a number of *crr* mutants of *S. typhimurium* and characterized these mutants with respect to III^{Glc} function by using a specific rabbit anti-III^{Glc} serum raised against purified III^{Glc} (33).

MATERIALS AND METHODS

Bacterial strains. The strains of *S. typhimurium* used in this study are listed in Table 1. The phenotypic characteristics of representative strains have been described previously (31).

Media and growth conditions. Cells were grown at 37°C on a rotary shaker either in liquid medium A (31) supplemented with a carbon source (0.2%) and 20 μg of the required amino acid per ml or in nutrient broth (0.8% nutrient broth [Difco Laboratories, Detroit, Mich.] supplemented with 0.5% NaCl). Transductions involving Tn*10* were performed on nutrient agar plates (nutrient broth solidified with 1.5% agar) containing 25 μg of tetracycline per ml.

Isolation of mutants. *crr* Δ(*ptsHI*) double mutants were isolated from either strain SB2309 or strain PP881 by spontaneous reversion on minimal salts agar plates containing 0.2% maltose. Mutations that enabled a *ptsHI* deletion strain to grow on maltose, melibiose, and glycerol and were cotransducible with *cysA* by using phage P22 were designated *crr*.

The isolation of strain PP386 (*crr-306*) has been described previously (31). Strains PP378 (*crr-303*) and PP385 (*crr-304*) were isolated in the same way. Strains PP780 (*crr-303*) and PP782 (*crr-306*) were constructed as described previously (31).

Strain PP888 (*crr-3*) was constructed by crossing phage P22 grown on a strain which contained the *crr-3* mutation (28) with cells of strain NK186 (*cys-*

TABLE 1. Origins and genotypes of *Salmonella* strains

Strain	Relevant genotype	Source or reference
SB3507	*trpB223*	E. Balbinder
	cysA20	P. E. Hartman
SB2309	*trpB223* Δ(*cysK-ptsHI*)*41*	2
PP642	Δ(*cysK-ptsHI*)*41*	31
SB2950	*trpB223* Δ(*cysK-ptsHI-crr*)*49*	2
PP800	*trpB223* Δ(*cysK-ptsHI-crr*)*49 galP283*	25
PP378	*trpB223* Δ(*cysK-ptsHI*)*41 crr-303*	This paper
PP385	*trpB223* Δ(*cysK-ptsHI*)*41 crr-304*	This paper
PP386	*trpB223* Δ(*cysK-ptsHI*)*41 crr-306*	31
PP994	*crr-307*::Tn*10*	This paper
PP780	*crr-303*	This paper
PP782	*crr-306*	31
PP888	*crr-3*	This paper
PP881	*cysA1539*::Tn*10* Δ(*cysK-ptsHI*)*41*	This paper
PP1180	*cysA1539*::Tn*10* Δ(*cysK-ptsHI*)*41 trpB223 crr-310*	This paper
PP1181	*cysA1539*::Tn*10* Δ(*cysK-ptsHI*)*41 trpB223 crr-311*	This paper
PP1182	*cysA1539*::Tn*10* Δ(*cysK-ptsHI*)*41 trpB223 crr-312*	This paper
PP1183	*cysA1539*::Tn*10* Δ(*cysK-ptsHI*)*41 trpB223 crr-313*	This paper
PP1184	*cysA1539*::Tn*10* Δ(*cysK-ptsHI*)*41 trpB223 crr-314*	This paper
PP1185	*cysA1539*::Tn*10* Δ(*cysK-ptsHI*)*41 trpB223 crr-315*	This paper
PP1186	*cysA1539*::Tn*10* Δ(*cysK-ptsHI*)*41 trpB223 crr-316*	This paper
PP1133	*ptsM416 trpB223*	This paper
PP1125	*trpB223 ptsG204 ptsM416*, Tn*10* near *ptsM*	22
PP1237	*ptsM416 galP283 trpB223* Δ(*cysK-ptsHI-crr*)*49*	This paper
PP1153	*ptsM416 crr-306*	This paper
PP1295	F'198/*cysA20*	This paper
PP1240	F'198/*crr-306 cysA1539*::Tn*10*	This paper
NK186	*cysA1539*::Tn*10*	J. Roth

A1539::Tn*10*) on galactose minimal salts plates lacking cysteine. *crr* transductants were recognized by their inability to grow on succinate, xylose, and citrate (31). *ptsM* Δ(*ptsHI-crr*)*49* and *ptsM crr* strains were constructed by using phage P22 to introduce the *ptsM416* mutation of strain PP1125 into strains PP800 [Δ(*ptsHI-crr*)*49 galP trpB223*] and PP782 (*crr-306*) respectively, and selecting for resistance to tetracycline and slow growth on galactose, which is transported by the II-A/II-B pathway in these strains (22). Strains PP1180 through PP1186 were isolated by crossing phage P22 grown on independent *crr* derivatives of strain PP881 with strain SB3507 on nutrient agar supplemented with tetracycline and selecting mannitol$^-$ maltose$^+$ transductants. Strain PP881 was isolated by crossing phage P22 grown on strain NK186 with strain PP642 and selecting for tetracycline-resistant transductants that still contained the *ptsHI* deletion. Strains PP1295 and PP1240 were constructed by crossing F'198/FF7040 (obtained from J. Lengeler, University of Regensburg, Regensburg, Germany) with *cysA20* and strain PP816 (*crr-306 cysA1539*::Tn*10*) on minimal salts plates lacking cysteine and supplemented with 0.2% galactose.

Insertion of Tn*10* into the chromosome of *S. typhimurium* was performed as described by Davis et al. (3), using a P22 lysate of strain NK337 [*hisC527 leu-414 supE* (P22 *c*2ts29 12amN1 13amH101 *int*-3 Tn*10*)], which was a generous gift from J. Roth. The *crr*::Tn*10* mutation was obtained by inserting Tn*10* into strain PP642 [Δ(*ptsHI*)*41*] on maltose minimal salts agar plates supplemented with tetracycline. One tetracycline-resistant strain with a *ptsHI crr* phenotype was isolated, and the Tn*10* insertion in this strain was cotransducible with *pts* and *cysA*. Strain PP994 was obtained by crossing the Tn*10* insertion into strain SB3507 and selecting for tetracycline resistance. Strain PP994 had a *crr* phenotype; i.e., it did not grow on succinate or xylose (31). Cotransduction between tetracycline resistance and the *crr* phenotype was 100% (96 of 96).

Immunochemical procedures. A specific rabbit anti-III^{Glc} serum was prepared as described previously (33). Rocket electrophoresis, crossed immunoelectrophoresis, and double-diffusion immunoprecipitation were performed as described previously (33). The gel systems routinely used contained 38 mM Tris, 100 mM glycine (pH 8.7), 1% Triton X-100, and 1% agarose.

Preparation of cell-free (crude) extracts and in vitro PTS assay. Cells were ruptured by passage through a French pressure cell, and crude extracts were prepared as described previously (31). The soluble protein fractions (supernatants) and membrane fractions were obtained by centrifugation of a crude extract at 200,000 × *g* for 90 min at 4°C. All preparations were made in a buffer containing 50 mM potassium phosphate, 1 mM EDTA, and 1 mM dithiothreitol (Sigma Chemical Co., St. Louis, Mo.); the final pH was 7.5 unless stated otherwise. Phosphoenolpyruvate-dependent phosphorylation of ^{14}C-labeled substrates was measured by the ion-exchange method (35). The preparation of partially purified enzyme I and HPr and the preparation of purified III^{Glc} have been described previously (33). The batch of III^{Glc} which we used was obtained after elution on an octyl-Sepharose column;

the specific activity of this III^{Glc} was 900 nmol of methyl α-glucoside phosphorylated per min per mg at 37°C.

Partially purified IgG fractions. Partially purified immunoglobulin G (IgG) fractions were obtained as described by Nowotny (19). Briefly, whole serum was dialyzed extensively against 5 mM sodium phosphate (pH 7.0). Equilibrated DEAE-cellulose (Whatman DE-52) was added to the dialyzed serum (2 ml of slurry per ml of serum). IgG was found in the supernatant, which was concentrated by freeze-drying in the case of the preparation used in the experiment shown in Fig. 5.

Purification of III^{Glc} by affinity chromatography. An affinity column was obtained by coupling partially purified IgG prepared from 6 ml of specific antiserum to 1.5 g of CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden), using the procedure described by the manufacturer. This column (3.5 ml) was equilibrated with a buffer containing 10 mM sodium phosphate and 150 mM NaCl (pH 7.2) (PBS) and was stored at 4°C in PBS containing 1 mM sodium azide. III^{Glc} was purified from 200,000-×*g* supernatants of strains SB3507, PP1184, and PP1186, which were prepared in PBS. The cells were grown on nutrient broth overnight. The supernatants contained approximately 10 mg of protein per ml. The samples (5 ml of the strain SB3507 supernatant and 15 ml of the supernatants from the *crr* strains) were pumped through the column at a flow rate of 0.5 ml/min at 4°C. The column was washed with 30 ml of PBS containing 0.5 M KSCN. After passage through the column, no III^{Glc} was detected in the eluate. III^{Glc} was eluted with 20 ml of PBS containing 4 M KSCN. The III^{Glc}-containing fraction was dialyzed against 1 mM Tris-hydrochloride (pH 7.5) and was concentrated by lyophylization. The recovery of III^{Glc} after the whole procedure was approximately 40%, as determined by rocket electrophoresis. III^{Glc} obtained in this way was more than 50% pure, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Thin-layer gel filtration of III^{Glc}. On one side of a glass plate (10 by 10 cm), a 1.5-mm layer of 1% agarose in 38 mM Tris–100 mM glycine (pH 8.7) was poured to a length of 10 cm and a width of 3 cm. In the middle of this agar slab a 1-cm lane was cut out and this lane was filled along its whole length (10 cm) with a thick slurry of Sephadex G-150 superfine (Pharmacia) equilibrated with the Tris-glycine buffer. The III^{Glc} samples (total volume, 20 μl) contained Dextran Blue 2000 (Pharmacia) and bromophenol blue as molecular weight markers, and these samples were applied to the surface of the slurry with a hypodermic needle. The samples were eluted with the Tris-glycine buffer by setting the plates at an angle of 20° for 3 h while they were connected on both sides to buffer reservoirs with paper wicks. III^{Glc} was visualized by immunoelectrophoresis perpendicular to the direction of elution. The apparent molecular weights of the peaks observed were calculated from their migration distances and those of the markers, assuming that the elution volume of a peak was inversely related to its migration distance and using the K_{av} values specified by the manufacturer.

Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis was performed essentially as described by Laemmli (15).

Detection of III^{Glc} with antiserum after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Electrophoretic transfer of proteins to nitrocellulose filters (Schleicher & Schull, Dassel, Federal Republic of Germany; filter type BA85; pore size, 0.45 μm) was performed as described previously (34). After transfer, the filters were washed four times for 10 min in a rotating cylinder with 5 ml of a buffer containing 10 mM Tris-hydrochloride, 150 mM NaCl, and 0.05% Tween 20 (Tween buffer). The filters were then incubated with 5 ml of 1% antiserum in Tween buffer. After the filters were washed six times (10 min each) with 5 ml of Tween buffer, they were incubated with ^{125}I-labeled protein A (7×10^6 cpm/μg; 300,000 cpm) in 5 ml of Tween buffer. Protein A (Pharmacia) was labeled by the chloramine-T method (7). The filters were washed six times with Tween buffer and then subjected to autoradiography on Kodak X-Omat R type XR-1 film.

Chemicals. [*U*-^{14}C]methyl α-glucoside (184 Ci/mol) was obtained from The Radiochemical Centre, Amersham, England. D-[*U*-^{14}C]mannose (50 Ci/mol), [^{14}C]methyl β-D-thiogalactoside (45 Ci/mol), and ^{125}INa (17 Ci/mg) were obtained from New England Nuclear Corp. Boston, Mass.

Protein determination. Protein was determined by the method of Lowry et al. (17), using bovine serum albumin as a standard.

RESULTS

III^{Glc} content of *crr* mutants. *pts crr* double mutants were isolated by spontaneous reversion on glycerol minimal agar plates containing a *ptsHI* deletion strain of *S. typhimurium* which could not grow on maltose, melibiose, or glycerol. These double mutants had regained the ability to grow on maltose, melibiose, and glycerol but not the ability to grow on succinate (23, 31). Since suppressors other than *crr* can yield the same phenotype (31), the location of the *crr* mutation near *cysA* (2) was checked by phage transduction in all cases. For instance, the *crr-303* mutation of strain PP386 was 34% cotransducible with *cysA*, whereas *pts* was 50% cotransducible in this cross. In some experiments we used strain PP881, which contained a *cysA*::Tn*10* mutation in addition to a *ptsHI* deletion. Phage P22 was grown on maltose$^+$ derivatives of this strain and was crossed with wild-type cells, selecting for tetracycline resistance. Transductants with a *cysA*::Tn*10* Δ(*ptsHI*) *crr* phenotype were purified and used for further study. The amount of III^{Glc} produced by strains carrying a *crr* mutation was measured by rocket immunoelectrophoresis (Table 2). All *crr* strains tested had lowered levels of III^{Glc} (i.e., 0 to 30% of the wild-type levels), as calculated from relative peak heights in rocket electrophoresis. Both a *ptsHI crr* deletion strain (SB2950) and a *crr*::Tn*10* strain (PP994), which apparently carried the Tn*10* insertion in the *crr* gene, contained no III^{Glc} detectable by rocket electrophoresis (Table 2). We considered the possibility that *crr*

TABLE 2. III^{Glc} in wild-type and mutant cells as determined by rocket electrophoresis[a]

Strain	Relevant genotype	III^{Glc} content (%)
SB3507	*trpB223 pts*$^+$	100
	cysA20 pts$^+$	100
SB2309	Δ(*ptsHI*)*41*	100
SB2950	Δ(*ptsHI-crr*)*49*	0
PP888	*crr-3*	20
PP780	*crr-303*	0
PP782	*crr-306*	0
PP994	*crr-307*::Tn*10*	0
PP1295	F'198/*cysA20*	300
PP1240	F'198/*crr cysA*::Tn*10*	200
PP378	Δ(*ptsHI*)*41 crr-303*	0
PP386	Δ(*ptsHI*)*41 crr-306*	0
PP1180	Δ(*ptsHI*)*41 crr-310*	5
PP1181	Δ(*ptsHI*)*41 crr-311*	5
PP1182	Δ(*ptsHI*)*41 crr-312*	0
PP1183	Δ(*ptsHI*)*41 crr-313*	5
PP1184	Δ(*ptsHI*)*41 crr-314*	30
PP1185	Δ(*ptsHI*)*41 crr-315*	0
PP1186	Δ(*ptsHI*)*41 crr-316*	20

[a] Cells were grown overnight on nutrient broth. After the cells were harvested and lysed with toluene, chloroform, and Triton X-100 (16), the amount of antigenic material which reacted with our antiserum was determined by rocket electrophoresis. The apparent III^{Glc} contents were determined from the peak heights after correcting for differences in protein contents between the samples, setting the value for wild-type cells at 100%; 100% corresponded to approximately 2.5 μg of wild-type III^{Glc} per mg (dry weight) (33).

strains containing low amounts of III^{Glc} represented a class of mutants that were genetically distinct from *crr* strains without detectable III^{Glc} as determined by immunoprecipitation. This could not be confirmed by cotransduction experiments with phage P22 grown on strains PP386 and PP1186 due to the statistical error inherent in this method (12). The *crr* mutations in these strains were linked closely in cotransduction; i.e., they were not more than two genes of average length apart, but we could not establish whether these mutations were located within the same cistron (data not shown).

Properties of mutant III^{Glc}. For further study we selected two Δ(*ptsHI*) *crr* strains with lowered levels of III^{Glc}, strains PP1186 and PP1184 (Table 2). The III^{Glc} produced by strains PP1184 and PP1186 could not be distinguished from wild-type III^{Glc} with respect to molecular weight (see Fig. 7) and isoelectric point (data not shown). After isoelectric focusing and blotting on nitrocellulose filters (33) of 200,000-×-*g* supernatants obtained from mutant and wild-type cells,we detected two main bands with specific anti-III^{Glc} serum at pH 4.0 and pH 3.9. Purified III^{Glc} showed the same behavior. III^{Glc} of wild-type and mutant cells was purified by using affinity chromatography, as described above. In double-diffusion immunoprecipitations, the III^{Glc} isolated from the mutant strains and the III^{Glc} isolated from wild-type cells behaved identically (Fig. 2). This showed that the *crr* mutations caused no major immunochemical changes in III^{Glc}. The enzymatic activities of the preparations were determined by measuring methyl α-glucoside phosphorylation in vitro. The III^{Glc} produced by the two *crr* strains was as effective in phosphorylation as wild-type III^{Glc} (Table 3). Apparently, the *crr* mutations of the mutants tested did not affect the enzymatic activity of III^{Glc}, but these mutations did lower the intracellular levels of III^{Glc}. As reported previously, III^{Glc} behaves heterogeneously in crossed immunoelectrophoresis (33) (Fig. 3). III^{Glc} from wild-type cells produced two peaks in one continuous precipitation line if electrophoresis in the first dimension was performed at 15°C (Fig. 3A). At 4°C an extra component which had a low mobility appeared (Fig. 3B). III^{Glc} isolated from strain PP1186 produced an extra peak compared with wild-type III^{Glc} at 15°C (Fig. 3C). At 4°C the two peaks with high mobilities almost disappeared, whereas a large slow-moving peak was visible (Fig. 3D). III^{Glc} isolated from strain PP1184 also showed altered electrophoretic behavior in crossed immunoelectrophoresis, but it produced less of the slow component compared with wild-type III^{Glc} (data not shown). The electrophoretic heterogeneity of III^{Glc} under these conditions was due to formation of multimeric complexes, as shown by thin-layer gel filtration followed by immunoelectrophoresis (Fig. 4). After gel filtration at room temperature of III^{Glc} from wild-type cells (Fig. 4A) and strain

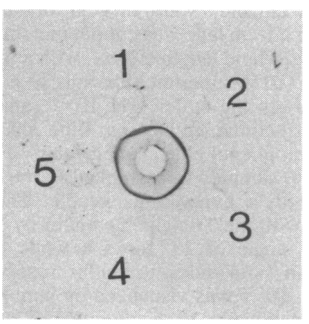

FIG. 2. Double-diffusion immunoprecipitation of III^{Glc}. III^{Glc} was purified by affinity chromatography from wild-type cells (strain SB3507) and from two *crr* mutants, strains PP1184 and PP1186. The center well contained 1.5 μl of anti-III^{Glc} serum, and each numbered well contained approximately 0.1 μg of III^{Glc}. Well 1, Strain PP1184; well 2, strain SB3507; well 3, strain SB3507; well 4, strain PP1186; well 5, strain SB3507.

TABLE 3. Specific enzymatic activities of wild-type and mutant III^{Glc}[a]

Source of III^{Glc} (strain)	Sp act (nmol/min per mg)
SB3507	600
PP1184	540
PP1186	600

[a] III^{Glc} was purified by affinity chromatography. The values for phosphoenolpyruvate-dependent methyl α-glucoside phosphorylation (1 mM) by the preparations were measured in vitro by using purified enzyme I and HPr and washed membranes from strain SB3507 as a source of IIB^{Glc}. The amount of III^{Glc} in each assay mixture was measured by using rocket electrophoresis and purified III^{Glc} as a standard (33).

PP1186 cells (Fig. 4B), we distinguished four components in both cases, but these components were present in different relative amounts. The apparent molecular weights of the components were 20,000, 40,000, 60,000, and 120,000. This suggested that under these conditions III^{Glc} formed di-, tri-, and hexameric complexes. We concluded from Fig. 4 that III^{Glc} isolated from strain PP1186 tended to form fewer high-molecular-weight complexes than wild-type III^{Glc}. This explained the results obtained with crossed immunoelectrophoresis. The altered electrophoretic behavior of mutant III^{Glc} was probably due to a mutation in the structural gene of III^{Glc}, but other possibilities cannot be excluded until decisive evidence concerning the location of the structural gene is obtained.

III^{Glc} activity in strains containing no soluble III^{Glc}. The *crr-303* mutant which contained no III^{Glc} detectable by immunoelectrophoresis (Table 2) was still able to take up methyl α-glucoside (24), whereas a *ptsG* mutant which lacked enzyme II-B^{Glc} was not (32). Crude extracts of such *crr* strains still showed considerable methyl α-glucoside phosphorylation in vitro. We determined the inhibition of this activity by the specific antiserum in order to establish whether it was due to III^{Glc}. This experiment was performed with strains which also carried a *ptsM* mutation in order to eliminate residual phosphorylation of methyl α-glucoside by IIA/IIB, which is independent of III^{Glc} (Fig. 1). Furthermore, we used a partially purified IgG fraction to minimize nonspecific inhibition. Methyl α-glucoside phosphorylation of a crude extract from *ptsM crr*$^+$ cells was inhibited more than 90% by anti-III^{Glc} IgG, whereas a preparation of preimmune IgG had no significant effect (Fig. 5A). Mannose phosphorylation by a crude extract of a *crr ptsM*$^+$ strain was insensitive to both anti-III^{Glc} IgG and preimmune IgG (Fig. 5D). Methyl

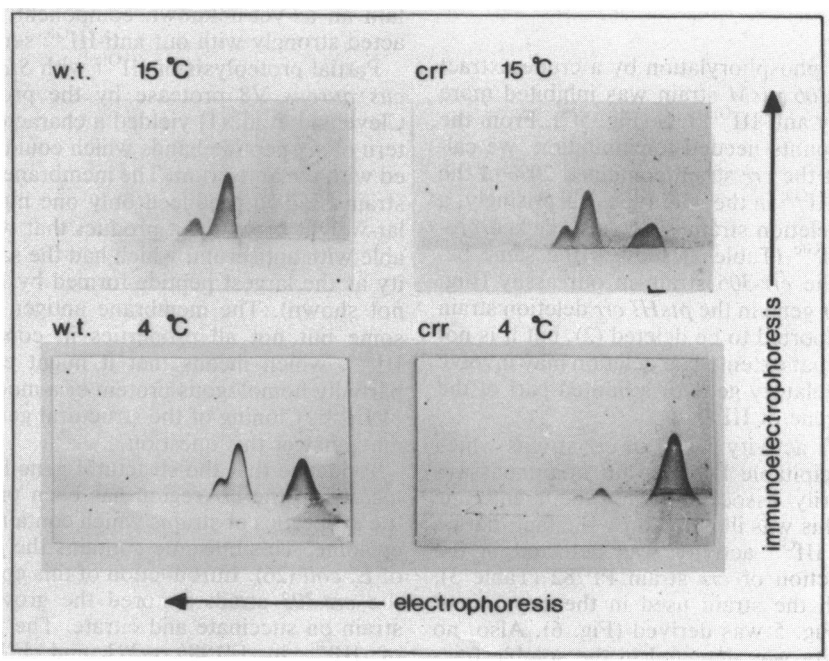

FIG. 3. Crossed immunoelectrophoresis of purified III^{Glc}. III^{Glc} was purified by affinity chromatography from strains SB3507 (wild type [w.t.]) and PP1186 (*crr-316*). In each case approximately 0.6 μg was applied to the gel and subjected to crossed immunoelectrophoresis. Electrophoresis in the first dimension was performed at either 4 or 15°C for 2 h. Immunoelectrophoresis was performed at 15°C overnight.

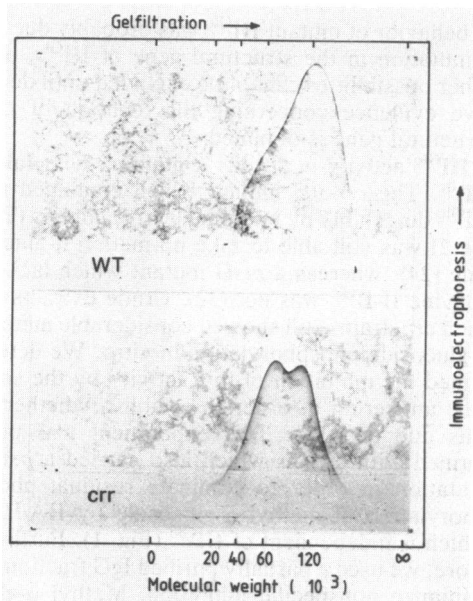

FIG. 4. Gel filtration of III^{Glc}. III^{Glc} was purified by affinity chromatography from strains SB3507 (wild type [wt]) and PP1186 (*crr-316*). Approximately 0.7 μg of each preparation was subjected to thin-layer gel filtration in Sephadex G-150 at room temperature, followed by immunoelectrophoresis at 15°C. The gel for the second dimension contained 0.75% antiserum. Apparent molecular weights are indicated on the horizontal axis.

α-glucoside phosphorylation by a crude extract from a *crr-306 ptsM* strain was inhibited more than 90% by anti-III^{Glc} IgG (Fig. 5C). From the relative amounts needed for inhibition, we calculated that the *crr* strain contained 20% of the amount of III^{Glc} in the wild type. Surprisingly, a *ptsHI crr* deletion strain which also lacked precipitable III^{Glc} (Table 2) showed the same behavior as the *crr-306* strain in our assay (Fig. 5B). The *crr* gene in the *ptsHI crr* deletion strain has been reported to be deleted (2), but it is not known to what extent. The deletion may involve either a regulatory gene or a limited part of the structural gene of III^{Glc}.

The III^{Glc} activity found in *crr* strains which lacked precipitable III^{Glc} in our immunoassays was primarily associated with the membrane fraction. This was illustrated by the fact that no significant III^{Glc} activity was detected in the soluble fraction of *crr* strain PP782 (Table 3), from which the strain used in the experiment shown in Fig. 5 was derived (Fig. 6). Also, no III^{Glc} activity was detected in the soluble fraction of the *ptsHI crr* deletion strain (2; Scholte, unpublished data). It should be noted that membrane-bound methyl α-glucoside phosphorylation activity has also been reported for wild-type cells (33), which indicates that this is not a peculiarity of *crr* mutants. The observed association of III^{Glc} activity with the membranes could explain our failure to detect the protein by immunoprecipitation. Therefore, we used a gel blotting technique (34) to visualize III^{Glc} by labeling the protein with specific antiserum and ^{125}I-labeled protein A after sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transfer of the proteins to nitrocellulose filters. High-speed supernatants and membranes of wild-type cells and a number of *crr* mutants were prepared and subjected to this treatment. On the autoradiogram purified III^{Glc} and supernatant from wild-type cells showed one band, with an apparent molecular weight of 21,000, as expected (Fig. 7, lanes 5 and 9). Wild-type membranes produced another strong band, which had a slightly higher mobility (Fig. 7, lane 6). Supernatant from strain PP1186 contained less III^{Glc} (Fig. 7, lane 3), whereas the membrane fraction produced a band of III^{Glc} and also a band of the membrane-bound antigen (Fig. 7, lane 4). In supernatants of strains PP782 (*crr-306*) and SB2950 [Δ(*ptsHI-crr*)*49*] we observed no III^{Glc} (Fig. 7, lanes 1 and 7), but the membranes did produce a strong band (Fig. 7, lanes 2 and 8). These results confirmed the conclusions of the rocket electrophoresis experiment with respect to the amount of soluble III^{Glc} in the cells (Table 2). However, the membranes appeared to contain an as-yet-unknown component, which reacted strongly with our anti-III^{Glc} serum.

Partial proteolysis of III^{Glc} with *Staphylococcus aureus* V8 protease by the procedure of Cleveland et al. (1) yielded a characteristic pattern of six peptide bands which could be detected with the antiserum. The membrane antigen of strain SB2950 produced only one high-molecular-weight breakdown product that was detectable with antiserum, which had the same mobility as the largest peptide formed by III^{Glc} (data not shown). The membrane antigen may have some but not all properties in common with III^{Glc}, which means that it might either be a partially homologous protein or a modified form of III^{Glc}. Cloning of the structural gene of III^{Glc} may answer this question.

Evidence that the structural gene for III^{Glc} is located in the *crr* region has been provided by the properties of strains which contain the F'198 episome. This episome contains the *pts* operon of *E. coli* (26). Introduction of this episome into the *crr-303* strain restored the growth of this strain on succinate and citrate. The production of III^{Glc} in F'198/*crr-303* and F'198/*cysA20* strains was proportional to the *crr* gene copy-number (Table 2).

Although we were not able to discriminate between *E. coli* III^{Glc} and *Salmonella* III^{Glc} (33),

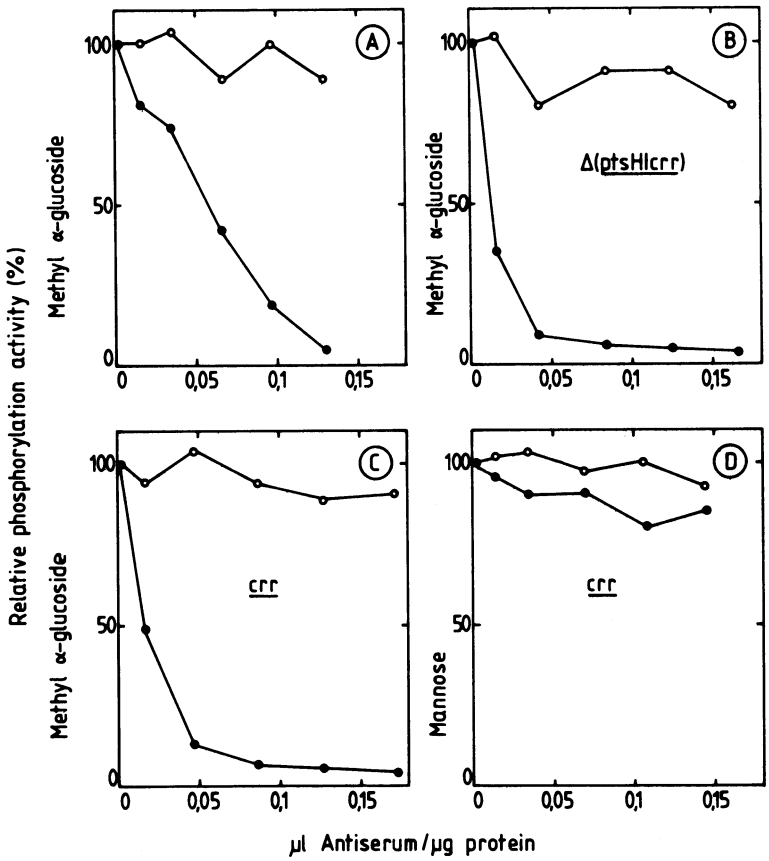

FIG. 5. III^{Glc} activities in high-speed supernatants. Phosphoenolpyruvate-dependent phosphorylation by crude extracts of 1 mM [^{14}C]methyl α-glucoside (A through C) or 10 mM [^{14}C]mannose (D) was measured in vitro as described in the text. Then 0.004 IU of partially purified enzyme I and HPr was added per 100 µg of crude extract to ensure that III^{Glc} activity was rate limiting. Reaction rates are expressed as percentages of the uninhibited value. A partially purified IgG fraction of either preimmune serum (○) or specific anti-III^{Glc} serum (●) was added in different amounts to the complete reaction mixture, and the preparation was incubated for 60 min. The reaction was started by adding radioactive substrate. (A) Strain PP1133 (*ptsM*). 100% = 3 nmol/min per mg. (B) Strain PP1237 [*ptsM* Δ(*ptsHI-crr*)]. 100% = 5 nmol/min per mg. (C) Strain PP1153 (*ptsM crr*). 100% = 4 nmol/min per mg. (D) Strain PP782 (*crr*). 100% = 13 nmol/min per mg.

we concluded that the structural gene is located in the *crr* region.

DISCUSSION

We isolated *crr* mutants of *S. typhimurium* by using the following two criteria: (i) a *crr* mutation permits growth of a *ptsHI* deletion strain on melibiose, glycerol, and maltose; and (ii) a *crr* mutation is cotransducible with *cysA*. All *crr* mutants which we tested showed a lowered content of III^{Glc}, as determined by rocket immunoelectrophoresis (Table 2). This is in accordance with earlier reports which showed that *crr* mutants of *S. typhimurium* have lowered III^{Glc} activities (2, 28). The two classes of *crr* mutations that could be distinguished on the basis of their effects on the III^{Glc} contents of the cells (Table 2) may represent either mutations in two different but closely linked genes or mutations within one gene.

The III^{Glc} produced by strains PP1184 and PP1186 was purified and characterized in order to reveal the nature of the *crr* mutation. We found that the *crr* mutations which we studied had no significant effect on the enzymatic activity of III^{Glc} in vitro (Table 3). The molecular weight (Fig. 7), the isoelectric point (data not shown), and the immunochemical properties (Fig. 2) of III^{Glc} were also not altered to a measurable extent. A prominent feature of both wild-type III^{Glc} and mutant III^{Glc} was the tendency of these compounds to behave inhomogeneously in crossed immunoelectrophoresis (Fig. 3). This electrophoretic inhomogeneity was

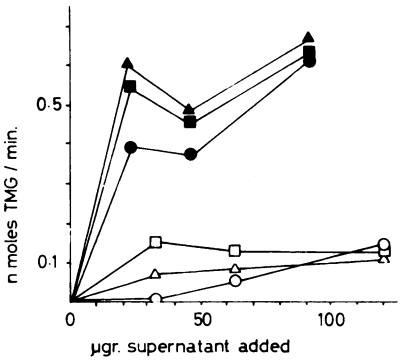

FIG. 6. III^{Glc} activities in soluble fractions. The III^{Glc} activities in 200,000 × *g* supernatants of crude extracts from strains SB3507 (closed symbols) and PP782 (open symbols) were measured as phosphoenolpyruvate-dependent phosphorylation of 5 mM [^{14}C]methyl β-D-thiogalactoside (TMG) (2). Different amounts of supernatants were assayed in the presence of 90 µg of washed membranes from strain SB3507 and varying amounts of purified enzyme I and HPr. Symbols: ● and ○, no enzyme I and HPr added; ■ and □, 0.008 IU of enzyme I and HPr added; ▲ and △, 0.016 IU of enzyme I and HPr added.

temperature dependent (Fig. 3) and correlated with inhomogeneity in gel filtration (Fig. 4). From this we concluded that III^{Glc} monomers tend to form polymeric structures. A similar behavior has also been reported in the case of III^{Lac} of *S. aureus*, which is functionally related to III^{Glc} (10). III^{Glc} isolated from strain PP1186 formed relatively fewer high-molecular-weight complexes than wild-type III^{Glc} at pH 8.6 (Fig. 4). III^{Glc} from strain PP1184 showed the opposite behavior (data not shown). The altered tendency to aggregate may have been caused by a structural mutation in III^{Glc}. A structural mutation could also explain the fact that *crr* mutants have lowered amounts of III^{Glc}, since it has been reported that defective proteins are degraded at a high rate in *E. coli* and *S. typhimurium* (6, 18). This implies that the gene affected in strain PP1186 is the structural gene for III^{Glc}. This conclusion is supported by the observation that strains which contain the F′ 198 episome of *E. coli* (Table 2) have elevated levels of III^{Glc}, suggesting that the structural gene of III^{Glc} is indeed localized in the *crr* region.

crr mutants that appeared to be devoid of III^{Glc} as measured by rocket immunoelectrophoresis still contained approximately 20% of the wild-type level of III^{Glc}, as shown by their antiserum-sensitive phosphorylation activity in vitro (Fig. 5). Surprisingly, strain SB2950, which has been reported to contain a deletion of the *crr* gene (2), behaved similarly (Table 2 and Fig. 5). Moreover, strain PP994, which appears to have a Tn*10* insertion within the *crr* gene, also lacked precipitable III^{Glc} (Table 2), but it still had about 20% of the wild-type methyl α-glucoside transport activity (data not shown). The phosphorylation activity of the *crr* strains which lacked precipitable III^{Glc} was strongly associated with the membranes (Fig. 5 and 6). It seems rather unlikely that the deletion in strain SB2950 and the insertion in strain PP994 affect the structural gene of the protein which is responsible for the methyl α-glucoside phosphorylation activity in these strains. An alternative explanation is that there are two genes in the *crr* region which are involved in the production of III^{Glc}, namely, a structural gene, which may be altered in strains PP1184 and PP1186, and a regulatory gene, which may be affected in the *crr* strains that are devoid of precipitable III^{Glc}. However, it should be noted that III^{Glc} behaves like a constitutive protein in *S. typhimurium* (33). Therefore, no physiological function of the hypothetical regulator gene can be envisaged as yet.

A mutation in a regulatory gene can explain the low levels of III^{Glc} activity in *crr* strains but cannot explain the extent of the association of this activity with the membranes. A possibility that can be considered is that the phosphorylation activity found in *crr-306* and SB2950, although sensitive to specific anti-III^{Glc} serum

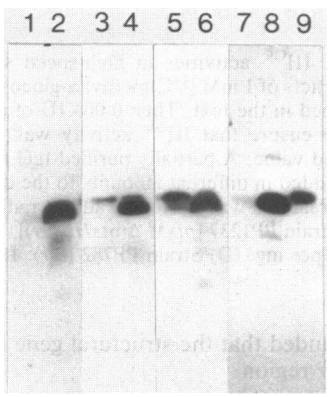

FIG. 7. III^{Glc} in soluble and membrane fractions from wild-type cells and *crr* mutants. We subjected 200,000 × *g* supernatants and membranes of different strains to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The antigenic determinants of III^{Glc} were visualized by labeling with specific antiserum and ^{125}I-labeled protein A after transfer to nitrocellulose filters. Approximately 50 µg of each preparation was applied to each gel. Lane 1, strain SB2950 supernatant; lane 2, strain SB2950 membranes; lane 3, strain PP1186 supernatant; lane 4, strain PP1186 membranes; lane 5, strain SB3507 supernatant; lane 6, strain SB3507 membranes; lane 7, strain PP782 supernatant; lane 8, strain PP782 membranes; lane 9, purified III^{Glc} (0.5 µg).

(Fig. 5), is caused not by III^{Glc} but by a functionally and immunochemically related protein which is localized in the membrane. This possibility was suggested by the experiment shown in Fig. 7. The membranes of wild-type cells and *crr* mutants, including a *crr* deletion strain, contained a protein which cross-reacted with III^{Glc} when our specific antiserum was used (Fig. 7). This protein had a slightly higher mobility during sodium dodecyl sulfate-polyacrylamide gel electrophoresis than III^{Glc}. Also, it could be solubilized with different detergents but did not readily precipitate with our antiserum (data not shown). The nature of this membrane antigen is still being studied. One possibility is that the preparation of III^{Glc} against which the antiserum was raised may have been contaminated with membranes. This seems very unlikely since (i) a highly purified preparation was used (33) and (ii) purified III^{Glc} produced only one band on the autoradiogram (Fig. 7, lane 9). We could speculate that this antigen is either a modified form of III^{Glc} or a partially homologous protein. Such a partial immunochemical resemblance between products of different genes has been observed previously (for instance, in the case of three outer membrane proteins of *E. coli* [20]). The function of this protein is not yet clear, but it may be involved in PTS activity. This would explain the antiserum-sensitive and membrane-bound phosphorylation activity in strains with the *crr-303* mutation and the *ptsHI crr* deletion mutation, which appear to be devoid of precipitable III^{Glc} (Table 2 and Fig. 5 and 6). Cloning of the structural gene of III^{Glc}, which is in progress in our laboratory, may help us establish the nature of the membrane antigen. Moreover, analyses of these clones will help us decide whether a regulatory gene or a processing gene is involved in the synthesis of III^{Glc}.

In *E. coli, tgs* mutants which are defective in III^{Glc} can be obtained by selecting for resistance to 5-thioglucose. These mutants behave like our *crr* mutants with respect to phenotype and III^{Glc} activity (13, 14). The *crr* mutants of *E. coli* which were obtained by Kornberg et al. by spontaneous reversion of a *ptsI*^{ts} strain on maltose at 42°C appear to be defective in methyl α-glucoside transport. This can be inferred from the resistance of these mutants to methyl α-glucoside and 5-thioglucose (11, 32). These *crr* strains have also lost their preference for glucose over lactose and therefore are defective in catabolite repression. This class of *crr* mutants is apparently analogous to the *crr* mutants described in this paper. More recently, Kornberg et al. described a pleiotropic pseudorevertant of a *ptsI* strain, designated *ptsI*^{ts} *crr*, which has different characteristics (14). This *crr* mutant has normal methyl α-glucoside transport and phosphorylation rates, which indicates that the III^{Glc}/II-B^{Glc} pathway is intact. Growth of this *crr* strain on maltose was inhibited by 2-deoxyglucose but was insensitive to methyl α-glucoside, whereas growth on fructose was inhibited by both methyl α-glucoside and 2-deoxyglucose. These results are difficult to explain unless we assume that (i) deoxyglucose inhibits growth on maltose by a different mechanism than methyl α-glucoside and (ii) a factor other than III^{Glc} is involved in the inhibition. We have not been able to confirm this since we have only found *crr* mutants that are defective in III^{Glc}. Moreover, maltose uptake in these *crr* mutants is less sensitive to 2-deoxyglucose than maltose uptake in wild-type cells (S. Nelson et al., manuscript in preparation).

The study of PTS-mediated repression is complicated by the fact that this phenomenon is influenced by many parameters. Apart from *crr* mutants, several other classes of pleiotropic pseudorevertants of *ptsI* mutants can be obtained, among which are *crp** (31) and *cya** strains (Postma, unpublished data). Mutations which tend to activate repressed genes behave like *crr* mutations in some respects (Nelson et al., manuscript in preparation). The *crr* mutants described in this paper caused a decrease in the amount of III^{Glc} in the cells. This resulted in a partial relief of repression in a Δ(*ptsHI-crr*) strain and in a partial defect in adenylate cyclase activity in a *crr* strain (5, 31). This is consistent with the model in which III^{Glc} in the phosphorylated form acts as an activator of adenylate cyclase and in the unphosphorylated form acts as an inhibitor of certain transport systems (23, 28, 29). The involvement of other factors is not excluded, however. In the future this matter may be resolved by studying the binding of III^{Glc} to its possible targets.

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