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Biochemical Characterization of the *ctr* Mutants of *Escherichia coli*[1]

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A test procedure based on complementation in mixed extracts is described for the assay of heat-stable protein and enzyme I of the phosphoenolpyruvate-dependent phosphotransferase system. The test was used to assay a collection of pleiotropic carbohydrate mutants of *Escherichia coli* (*ctr* mutants) and revertants of these mutants. All mutants were found to lack enzyme I of the phosphoenolpyruvate-dependent transferase system. Revertants of these mutants to complete wild phenotype regained enzyme I-forming ability. Reversion to partial wild type was not accompanied by restoration of enzyme I-forming ability.

Complementation in extracts for the identification of *Escherichia coli* strains lacking the heat-stable protein (HPr) or enzyme I of the phosphoenolpyruvate (PEP)-dependent phosphotransferase system was reported briefly (9). The initial study has been extended to characterize partially many additional *ctr* mutants. The test is based on the β-glucoside catabolism scheme of Fox and Wilson (2), and we have isolated a mutant (*ctr-7*) in a strain of *E. coli* K-12 which, in the absence of any inducer, produces all of the components of this scheme except enzyme I. The absence of enzyme I in *ctr-7* has been verified by comparison with the known enzyme I-deficient strain MM6. The defect of *ctr-7* has been mapped by transduction in the vicinity of the mutation in MM6. A second test strain (1101) for the measurement of HPr was obtained from C. F. Fox. Strain 1101 in the presence of saligenin-β-D-glucopyranoside (salicin) produces all of the components of the catabolic scheme except HPr. Mixtures of extracts from strains deficient in either HPr or enzyme I will complement each other and release *o*-nitrophenol (ONP) when ONP-β-D-glucoside and PEP are added to the system.

MATERIALS AND METHODS

Cultures. Six derivatives of *E. coli* K-12 with the *ctr* mutation were described by Wang et al. (9). These are designated *ctr-1* through *ctr-6*. The HPr-deficient mutant 1101 was described by Fox and Wilson (2). It requires histidine for growth in minimal medium and can be induced to form phospho-β-D-glucosidase and the enzyme II for β-D-glucoside phosphorylation during growth in salicin. The strain *ctr-7* was derived from a spontaneous mutant of AT2570 (9) which utilizes salicin constitutively. No induction is necessary for the formation of the phosphoglucosidase and its specific enzyme II in *ctr-7*. Strain 2570 is the parent strain of *ctr-3*. Strain MM6 from Tanaka et al. (7) was described previously (9).

Media. LB broth (11) was used for the growth of all mutants and the tester strain *ctr-7*. For the induction of the β-D-glucoside utilization system in 1101, the HPr-deficient second tester strain, we used D(M) minimal medium (11) with 0.2% yeast extract and 0.5% sodium succinate. After overnight growth in this medium, the culture was diluted 20 times, and salicin was added at a concentration of 0.125%. Growth was continued an additional 4 to 5 hr at 37 C.

The cholate indicator medium of Morse and Alire (5) was used for selection of carbohydrate revertants and for phenotypic determination of the various strains.

Collection and storage of cells. A 2-liter flask containing 1 liter of medium was inoculated with a single Mtl^- colony from a cholate-mannitol plate. (Symbols: Mtl, mannitol; Tna, tryptophanase; Sal, salicin; Glp, glycerol; Glu, glucose; Fru, fructose; Man, mannose; Lac, lactose; Mal, maltose; Suc, inability to grow on succinate; Ace, inability to grow on acetate; HPr, heat-stable protein; EnzI, enzyme I of the phosphotransferase system.) Flasks were incubated for 16 to 18 hr to stationary phase of growth in a rotary flask shaker at 37 C. All cultures were checked for the presence of reversions by platings on mannitol-indicator agar plates and discarded if the number of revertants to Mtl^+ exceeded 1% (Mtl^+ being used as test for reversion to Ctr^+). The cell paste collected after centriguation was frozen and stored at least overnight, but not for more than 3 to 4 days.

Standard solutions. A standard tris(hydroxymethyl)

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aminomethane (Tris) buffer, consisting of 0.1 M Tris-hydrochloride, 0.05 M NaCl, and 0.05 M $MgCl_2$, was adjusted to *p*H 7.6. *o*-Nitrophenyl-β-D-glucopyranoside (ONP-β-G), obtained from Mann Research Laboratories, New York, N.Y., was suspended in this buffer to 2 × 10^{-2} M concentration. A 0.1 M cyclohexyl ammonium phosphoenolpyruvate solution was made in 0.1 M Tris buffer plus 0.05 M $MgCl_2$ and adjusted to *p*H 7.5 with NaOH.

Preparation of cell extracts. The frozen cell paste was weighed and suspended in the standard buffer with the addition of NaF to 0.1 M. One gram of cell paste per 2 ml of buffer gave a suspension of about 50 mg of protein per ml after sonic disruption. The protein content was determined for each preparation by the method of Lowry et al. (4). Mercaptoethanol was added to give a final concentration of 10^{-2} M, and deoxyribonuclease (1 μg/ml) was also added. Sonic disruption was carried out at setting 5 of a Branson model S75 sonifier for a total of 60 to 80 sec in 20-sec bursts. Tubes were immersed in a salt-ice bath during sonic treatment.

Test method. All complementation assays were run within 1 hr after preparation of the extracts. Extracts of the tester strains (*ctr-7* and 1101) were used in constant amounts (0.2 ml) for all tests. Samples of the extracts of the unknown strains were used in amounts varying up to 0.15 ml, and the total volume was adjusted to 0.35 ml by addition of standard Tris buffer plus NaF. Solutions of ONP-β-G (0.1 ml) and PEP (0.1 ml) were added immediately before mixing and incubation.

The reaction was carried out in a 37 C water bath for 10 min and was then stopped by addition of 2.0 ml of 0.25 M Na_2CO_3 per tube. Under these conditions, production of ONP by complementation is proportional to time for extract concentrations of 1 to 3 mg of protein per 2.55 ml of reaction mixture. The tubes were mixed, put on ice, and refrigerated overnight. Refrigeration allowed more complete clearing of solutions. On the next day, the tubes were centrifuged at 40,000 × *g* for 10 min, and the supernatant solution was read at 415 nm in a Beckman DB spectrophotometer against 0.25 M Na_2CO_3. Where necessary, dilutions were made in 0.25 M Na_2CO_3.

All cultures including the tester strains were checked for "leakiness" by running the extract alone (not complemented) with the substrate for various periods of time (0 to 60 min).

Two blanks were made for each sample tested. The first was made by running only the tester extract (0.2-ml amounts of *ctr-7* or 1101) plus buffer, ONP-β-G, and PEP under standard test conditions. The second was made by the incubation of varying amounts of the unknown samples under test conditions. Absorbance due to the blanks was subtracted from each value of the complemented samples. Finally, the readings were converted to micromoles of ONP released per 2.55 ml of reaction mixture. The tester extracts usually contained about 50 mg of protein per ml. The protein content of extracts was not measured or adjusted prior to complementation tests because of the lability of these extracts (3). Samples were removed, and protein concentration was determined after the testing.

RESULTS

Complementation test. The complementation test depends upon the synthesis of ONP-β-G-6-phosphate in extracts and its subsequent hydrolysis by a phosphoglucosidase to yield ONP. Theoretically, it should be possible to assay for each factor involved in the synthesis of this product, but we restricted our interest only to the assay of enzyme I and HPr of the phosphotransferase system. Figure 1 shows in vitro complementation to produce ONP in a mixed extract of an HPr^-EnzI^+ strain to which has been added varying amounts of an HPr^+Enzl^- strain. In this experiment, enzyme II for β-glucosides is also varied, as the $EnzI^-$ strain produces this enzyme constitutively, but the experiment demonstrates the feasibility of in vitro complementation. When an extract containing only HPr is varied, essentially the same results are obtained, except that the reaction plateaus at a lower value. The results indicate that, under these conditions (HPr^-EnzI^+ extract plus HPr^+EnzI^- extract), HPr is the limiting factor in ONP pro-

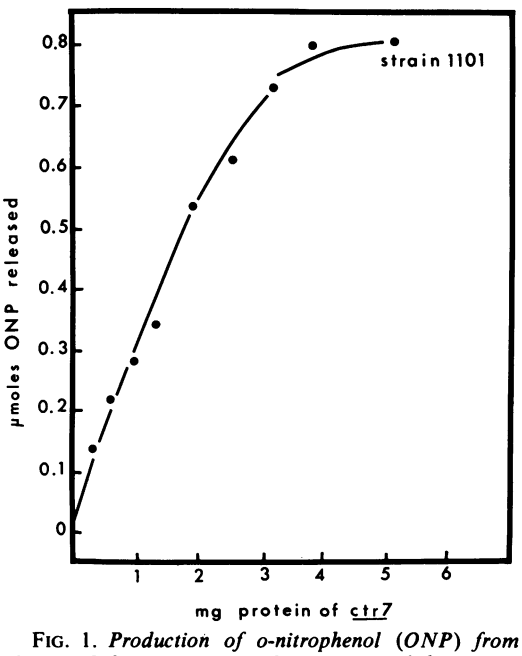

FIG. 1. *Production of o-nitrophenol (ONP) from ONP-β-G by in vitro complementation of the extract of strain 1101 (HPr-deficient) by increasing amounts of extract of strain ctr-7 (enzyme I-deficient). In this test, the production of ONP is followed as a function of the amounts of HPr and enzyme II for β-glucosides. When an extract of a strain lacking enzyme II for β-glucoside phosphorylation but containing HPr is added, essentially the same results are obtained, except that the production of ONP plateaus at about 0.3 to 0.5 μmole.*

duction over the range of 1 to 3 mg of the varied protein in the reaction mixture. Extracts of HPr$^+$ EnzI$^-$ strain (including *ctr-7*) can still complement after heating to 92 C for 3 min, indicating that these strains supply a heat-resistant factor.

Figure 2 shows the reciprocal experiment: the addition of varying amounts of HPr$^-$EnzI$^+$ extract to a constant amount of HPr$^+$EnzI$^-$ extract. Again complementation is observed, with proportionality over the range of 1 to 3 mg of added extract.

The tester strains by themselves do not produce ONP in amounts sufficient to interfere. Strain *ctr-7* itself (5 to 10 mg/reaction mixture) produces no ONP in 60 min; strain 1101 produces about 0.003 μmole of ONP per min under test conditions.

Most of the *ctr* mutants studied in this laboratory and considered below have been obtained in strains lacking the enzyme II for β-glucoside phosphorylation. Extracts of these mutants were incubated with PEP and ONP-β-G as a control when their complementation behavior was being assayed. Because they lack the specific enzyme II, they are not expected to be leaky, and this is what was found, with one exception. Mutant *ctr-2* for unexplained reasons produces about 0.05 μmole of ONP in 30 min under test conditions, an amount still insufficient to complicate testing.

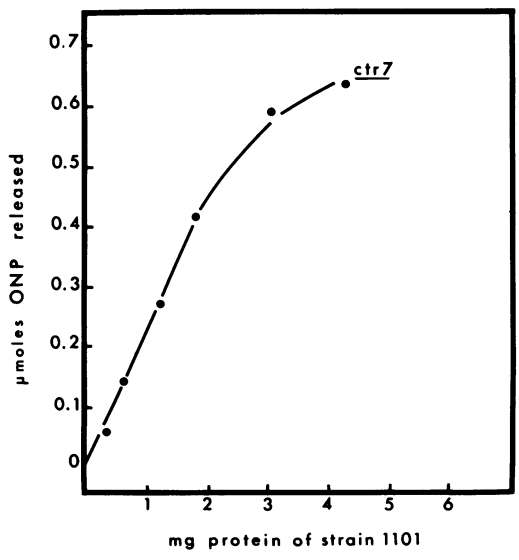

FIG. 2. *Production of o-nitrophenol (ONP) from ONP-β-G by in vitro complementation of the extract of strain ctr-7 (enzyme I-deficient) by increasing amounts of strain 1101 (HPr-deficient, but containing enzyme I and enzyme II for β-glucoside phosphorylation). When an extract containing enzyme I but lacking the enzyme II for β-glucosides is added, essentially the same results are obtained.*

In preparing the tester strains for the in vitro complementation assay, it would have been most desirable to have the mutations affecting enzyme I and HPr in a common background forming all of the other components for β-glucoside utilization constitutively. Considerable effort was expended to achieve this end. For example, the HPr mutation of strain 1101 was transferred by transduction to the strain utilizing β-D-glucosides constitutively. Unfortunately, of four such gene transfers, all hydrolyzed ONP-β-G to ONP in extracts. No explanation for this result is available. A reciprocal experiment was tried, the transfer of the constitutive utilization of β-D-glucosides from the parent strain of *ctr-7* to strain 1101. The linkage map of *E. coli* (8) indicates that the location of the gene regulating β-D-glucoside utilization is very close to the gene for tryptophanase production. A *tna* derivative of strain 1101 was isolated and used as a recipient for transductions from the constitutive strain, in the hope that the constitutive property might be cotransduced with Tna$^+$. (This could be tested for by complementation in extracts.) Of 12 transductants, none had received the ability to produce components for β-glucoside utilization constitutively. From this we conclude that either the genetic map positions are not as close as indicated or that the constitutive property in the parent strain of *ctr-7* is not *tna*-linked.

Analysis of ctr mutants by complementation with tester strains. Approximately 100 mutants with a phenotype resembling *ctr* mutants have been isolated in this laboratory. At least 17 of these mutants have been shown by transduction to be located at 46 to 47 min on the *E. coli* chromosomal map, where the mutation of strain MM6, a known enzyme I mutant, has also been located. At least 20 of these mutants, some with quite contrasting phenotypes, including the 17 above, have been analyzed by the complementation test.

Figure 3 shows the in vitro complementation behavior of *ctr* mutants 1 through 6, strains previously described (9). All produce ONP in extracts when mixed with the HPr$^-$EnzI$^+$ strain, and all fail to produce ONP when mixed with the HPr$^+$EnzI$^-$ *ctr-7* strain. It is concluded from these results that these strains, and the 14 others tested giving similar results, lack enzyme I of the phosphotransferase system.

Complementation testing of revertants of ctr. Genetic reversions of *ctr* mutants can be of two types: to complete restoration of the wild phenotype, or to one or another partial wild phenotype. The first of these classes has been found to occur at the *ctr* region (46 to 47 min); the others may be located at different places on the *E. coli*

chromosomal map, depending upon the phenotype (10). A number of these revertants have been analyzed by the in vitro complementation test (Table 1). Reversions to complete wild phenotype have restored enzyme I levels, but the various partial revertants examined still remain deficient in enzyme I.

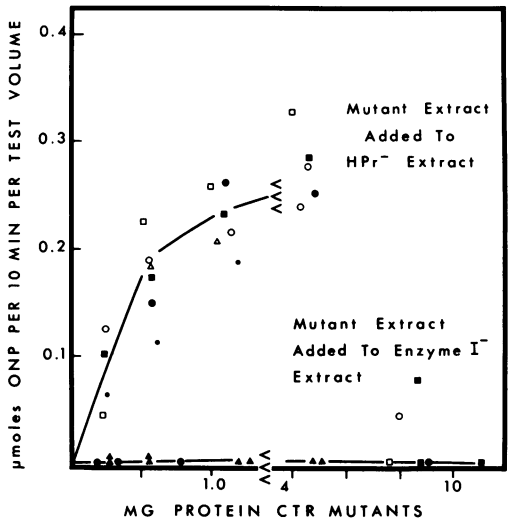

FIG. 3. *Testing of six ctr mutants, by in vitro complementation with strain 1101 (HPr-deficient) and strain ctr-7 (enzyme I-deficient). All mutants fail to complement ctr-7, but do complement strain 1101, showing that they contain HPr but lack enzyme I. A similar test employing strain MM6, a known enzyme I mutant, gives similar results.*

DISCUSSION

The in vitro complementation test previously outlined (9) is described in detail here. The test is relatively simple and sufficiently sensitive to be useful in classifying mutants defective in carbohydrate utilization. All of the *ctr* mutants examined failed to complement the *ctr-7* strain, which is not complemented by the MM6 strain, a strain known to be defective in enzyme I formation. The *ctr* mutants therefore must lack enzyme I. The basis for this deficiency is not known but could be twofold: a mutation in the structural gene for enzyme I; a mutation in a gene regulating enzyme formation.

Mutants with quite divergent phenotypes (Mtl⁻Sal⁻Glp⁻ as compared with Mtl⁻Sal⁻Glp⁻Glu⁻Fru⁻Man⁻Lac⁻Mal⁻Suc⁻Ace⁻) have been isolated which do not complement *ctr-7* and therefore must lack enzyme I. There does not appear to be a quantitative difference between these two types of strains in response in the complementation test, neither culture producing any ONP in 10 min at the highest concentrations of extract employed (ca. 8 mg of protein in the reaction mixture). Mutants with enzyme I defects might be expected to have different phenotypes if the formation of enzyme I protein were subject to regulatory gene control, and the regulatory gene also contributed to other aspects of metabolic regulation. Thus, mutation in the structural gene

TABLE 1. *In vitro complementation tests of selected ctr revertants*

Revertant phenotypes[a]	*ctr-7* Extracts			1101 Extracts		
	ctr-7 Protein (mg)	Revertant protein (mg)	ONP yield (μmoles)	1101 Protein (mg)	Revertant protein (mg)	ONP yield (μmoles)
Ctr⁺ (Fru)	2.0	9.6	1.2	2.0	9.6	0.28
Ctr⁺ (Mtl)	2.0	10.0	0.33	2.0	10.0	0.60
Ctr⁺ (Glu)	2.0	10.0	0.65	2.0	10.0	0.27
Ctr⁺ (Mel)	2.0	11.4	0.23	2.0	11.4	0.75
Lac⁺Glp⁺ (Lac)	8.0	11.8	0.0	6.5	11.8	0.95
Lac⁺Glu⁺ (Lac)	8.0	10.0	0.0	6.5	10.0	0.78
Glu⁺ (Glu)	8.3	11.0	0.0	8.0	11.0	0.52
Glp⁺Lac⁺Glu⁺ (Glp)	8.3	10.0	0.0	8.0	10.0	0.88
Glp⁺ (Glp)	8.3	11.8	0.0	8.0	11.8	0.43
Mel±Lac⁺Glp± (Mel)	8.9	10.8	0.0	8.3	10.8	0.37
Mel±Lac⁺Glp⁺Glu⁺ (Mel)	8.9	10.8	0.0	8.3	10.6	0.58
Mal⁺Glp⁺Mel⁺ (Mal)	8.9	10.0	0.0	7.8	10.0	0.63
Mal⁺Glp⁺Mel⁺ (Mal)	8.7	12.9	0.0	9.2	12.9	0.60
Mal⁺Glp⁺Mel⁺ (Mal)	8.8	13.3	0.0	9.2	13.3	0.50
Mal⁺Glp⁺Mel⁺ (Mal)	8.8	13.6	0.0	9.3	13.6	0.60
Mal⁺Glp⁺Lac⁺ (Mal)	8.2	10.0	0.0	7.3	10.0	0.23
Mal⁺Glp⁺Lac⁺ (Mal)	8.2	10.0	0.0	7.3	10.0	0.25

[a] Abbreviations in parentheses indicate the carbohydrate on which the revertant was selected. Abbreviations: Fru, fructose; Mtl, mannitol; Glu, glucose; Mel, melibiose; Lac, lactose; Glp, glycerol; Mal, maltose.

would result in loss of ability to phosphorylate salicin, fructose, mannitol, sorbitol, and mannose. Changes in a regulatory gene would affect the utilization of those carbohydrates phosphorylated by the phosphotransferase system and, in addition, any other cell properties governed by the regulatory gene. This model does not at the moment seem able to explain the phenotypes of the mutants with divergent phenotype that we have encountered. Saier, Simoni, and Roseman (6) presented preliminary evidence indicating organization of the genes for the phosphotransferase in *Salmonella typhimurium* into an operon, perhaps subject to a regulatory gene.

Partial reversions of *ctr* have been studied genetically. At least some of the Mal$^+$ reversions are located at *malB* (79 min); Lac$^+$ reversions are located at *lac* (11 min) (10). Recently we found the production of tryptophanase to be affected by *ctr* mutations and that partial reversion to Tna$^+$ occurs at *tna* (73 min) (1). The nature of the partial reversions remains to be clarified, but at least in the case of Lac$^+$ and Mal$^+$ it does not involve any change in enzyme I levels.

ACKNOWLEDGMENTS

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