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# 6-Phosphogluconolactonase Mutants of *Escherichia coli* and a Maltose Blue Gene

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Mutants lacking an enzyme of the oxidative branch of the hexose monophosphate shunt, 6-phosphogluconolactonase (*pgl*), have been selected as a new class of glucose-negative derivatives of a phosphoglucose isomerase (*pgi*) mutant. Glucose negativity is not as complete as in mutants lacking phosphoglucose isomerase and glucose-6-phosphate dehydrogenase. *Pgi*$^+$, *pgl*$^-$ strains have been constructed by transduction and grow almost normally on glucose. Genetic mapping shows that *pgl* lies between *chlD* and *att*-λ, in the same position as and identical with a *blu* gene described by Adhya and Schwartz. These *blu* mutants grown on maltose were recognized by their property to turn blue after treatment with iodine. It is not known how phosphogluconolactonase deficiency causes this reaction; it might be related to accumulation of 6-phosphogluconolactone.

In *Escherichia coli*, glucose is metabolized by the glycolytic pathway and the hexose monophosphate shunt (Fig. 1). The second step of the shunt is the hydrolysis of 6-phosphogluconolactone to gluconate-6-phosphate. An enzyme which catalyzes this reaction has been detected (2, 10) in several organisms. However, the reaction also goes spontaneously, so the physiological role of the enzyme is not understood.

In this paper we report the isolation and some characteristics of *E. coli* mutants lacking phosphogluconolactonase. The genetic map position of the mutations has been determined, and they are shown to be identical with a previously unexplained class of mutations causing the "maltose-blue" phenotype.

## MATERIALS AND METHODS

**Preparation of 6-phosphogluconolactone.** The substrate of the lactonase is 6-phosphoglucono-δ-lactone. This is not commercially available. We have used three preparative techniques: (i) bromine oxidation of glucose-6-phosphate (8); (ii) platinum-catalyzed oxidation of glucose-6-phosphate, adapted from a technique (3) for pyranose oxidation; and (iii) lactonization of gluconic acid 6-phosphate by lyophilization (10). Each method has usually given only about 25% yield (according to the hydroxamate assay, and assuming the extinction coefficient of the hydroxamate of 6-phosphogluconolactone to equal that of the hydroxamate of δ-gluconolactone). Infrared spectroscopy of the products (kindly aided by E. Simons) indicated that both γ and δ lactones might be present. We have usually used the product made by lyophilizing gluconic acid 6-phosphate. Barium salt of gluconate-6-phosphate (200 mg; Sigma Chemical Co., St. Louis, Mo.) was suspended in water and put on a column (1 by 15 cm) of Dowex-50 ($H^+$ form), and the water eluant was collected until no longer acidic (approximately 100 ml). The eluant was lyophilized, and the residue was dissolved in 5 ml of water. This material (approximately 50 μmoles of lactone per ml according to hydroxamate assay) was used the same day in assays.

**Assay of 6-phosphogluconolactonase.** Cells were grown in a broth containing minimal medium 63 supplemented with 1% tryptic digest (BBL) and 0.4% yeast extract (Difco; 4). Extracts were either prepared from 250-ml cultures in logarithmic growth by the protocol described earlier (7), or from 10-ml, stationary-phase cultures. In the latter case, after washing in saline the cells were resuspended in 2.0 ml of 0.1 M K-phosphate (*p*H 7) containing 1 mM dithiothreitol, and after sonication and centrifugation the supernatant fractions (approximately 0.5 mg of protein per ml) were used without further dilution in the lactonase assay. This assay was that of Brodie and Lipmann (2), which measures, by a hydroxamate test, the rate of disappearance of substrate from the incubation mixture. Substrate (2.0 μmoles, approximately 0.04 ml of the lyophilization product) was added to 0.5 mg of extract protein in 1 ml of 0.1 M K-phosphate buffer (*p*H 7), and the tube was then transferred to a 30 C bath. At 0, 10, and 20 min, 0.2-ml portions were transferred to centrifuge tubes containing 0.2 ml of 3.5 M NaOH and 0.2 ml of 3 M $NH_2OH$. After 2 min, 0.2 ml of 4 M HCl was added, the precipitated proteins were removed by centrifugation, and 0.2 ml of 0.37 M $FeCl_3$ in 0.1 M HCl was added to each supernatant fraction. Absorbance was read in a Lumetron model 401 colorimeter with a 540 filter. Because of spontaneous hydrolysis of the substrate, a control lacking extract was always done at the same time; such a control

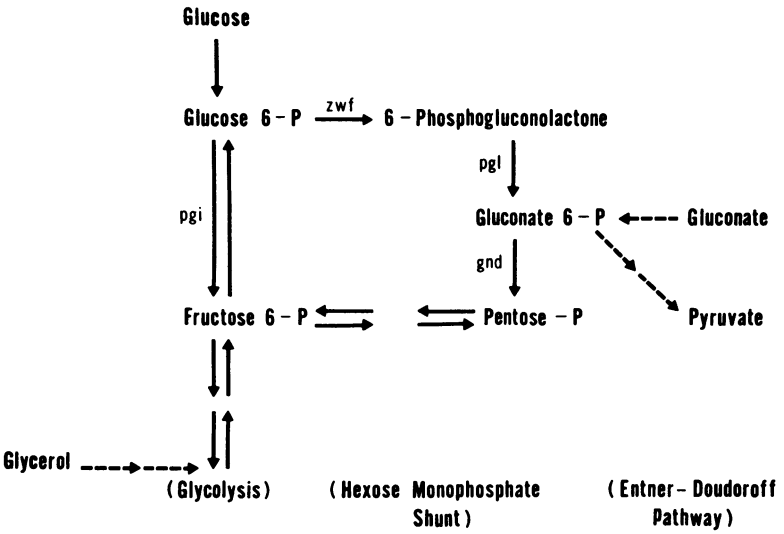


FIG. 1. *Pathways of glucose metabolism. Not all reactions are included. Dashed arrows represent inducible enzymes. Gene abbreviations are pgi for phosphoglucose isomerase, zwf for glucose-6-phosphate dehydrogenase, pgl for 6-phosphogluconolactonase, and gnd for gluconate-6-phosphate dehydrogenase.*

gave the same rate as one with boiled extract. A typical assay is illustrated in Fig. 2. A strain is scored as negative if the rate of disappearance of lactone is no faster in the presence of extract than in its absence; otherwise it is scored as positive. Because of the low sensitivity of the hydroxamate test and the appreciable spontaneous hydrolysis of the substrate, it was not possible to calculate specific activities of the lactonase by this assay. Nonetheless, it was satisfactory as a qualitative test, being reproducible and consistent with the growth phenotypes of all mutants and recombinants tested. A lactonase-negative extract did not inhibit the reaction of a positive extract. According to this test, the enzyme was present in lactonase-positive strains grown in various media, minimal (glucose, glycerol, or gluconate) as well as rich.

**Other assays.** Protein, glucose-6-phosphate dehydrogenase, gluconate-6-phosphate dehydrogenase, and phosphoglucose isomerase assays were described previously (7). Maltodextrin phosphorylase was measured by the method of Schwartz (11), and phosphoglucomutase by the method of Joshi and Handler (9).

**Media.** Most media have been described (7). TYE plates contained 10 g of tryptone (Difco), 5 g of yeast extract (Difco), 8 g of NaCl, and 15 g of agar per liter. EMB-maltose plates contained EMB agar base (Difco) and 0.8% maltose.

**"Blu" test.** This test was the slightly modified method of Schwartz (11). The cultures were patched to EMB-maltose plates. After 6 hr of incubation the plates were flooded with 4 ml of a solution containing 0.01 M $I_2$ and 0.03 M KI, and the patch color was visually scored as "blue" or "not blue". This test could also be done on isolated colonies, and minimal maltose or maltose + glycerol plates could be used instead of EMB-maltose (M. Schwartz, *personal communication*).

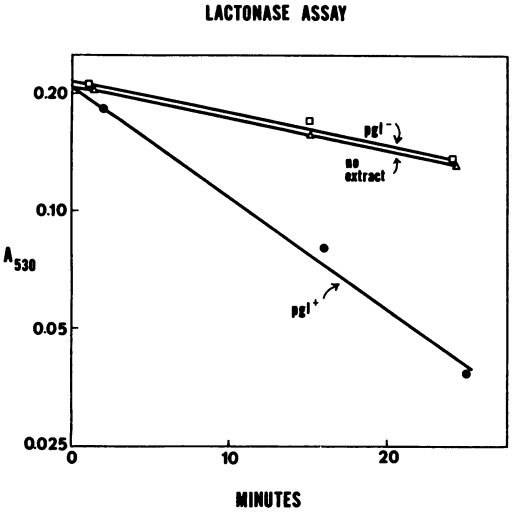


FIG. 2. *Lactonase assay. Details of the assay are given in the text. In this example the two strains were derived by conjugation of a str-r mutant of DF1311 (pgi⁻, pgl-1), used in phenocopy, with Hfr Hayes (pgi⁺, pgl⁺, str-s) and selection for glucose-positive, str-s recombinants.*

**Genetic techniques.** Conjugation and transductions were done by the method of Fraenkel (4). The presence of intact lambda prophage was tested by ultraviolet irradiation and replication onto a lambda-sensitive strain, CA85.

**Bacterial strains.** Strains are described in Table 1. Figure 3 shows the positions of some of the genetic markers employed.

TABLE 1. *Bacterial strains*

| Strain | Sex | Relevant markers[a] | str[b] | From |
|---|---|---|---|---|
| K-10 | HfrC | | s | This lab |
| DF40 | HfrC | *pgi-2* | s | (7) |
| DF1311 | HfrC | *pgi-2, pgl-1* | s | This paper |
| DF1312 | HfrC | *pgi-2, pgl-2* | s | This paper |
| DF2023 | HfrC | *pgi-2, pgl-3* | s | This paper |
| DF1070 | HfrC | *edd-1, gnd-1* | s | (5) |
| DF1071 | HfrC | *gnd-1* | s | (5) |
| DF2000 | HfrC | *pgi-2, zwf-2* | s | (5) |
| DF2001 | HfrC | *zwf-2* | s | (5) |
| X33 | F⁻ | *xyl⁻, his⁻, purE⁻, pyrF⁻, trp⁻* | r | J. Beckwith |
| DF402 | F⁻ | *his⁻, purE⁻, pyrF⁻, trp⁻, pgi-2* | r | —[c] |
| X7099 | F⁻ | *arg⁻, bioA⁻, his⁻* | r | J. Beckwith |
| DF403 | F⁻ | *bioA⁻, his⁻, pgi-2* | r | —[d] |
| SA500 | F⁻ | *his⁻, su⁻*, lambda-cI857 | r | S. Adhya |
| SA311, 315, 320 | F⁻ | *his⁻, chlD*-deletions | r | SA500, S. Adhya |
| SA194, 195, 197 | F⁻ | *his⁻, blu* | r | *blu* point mutants from SA500, S. Adhya |
| SA516, 517, 515 | F⁻ | *his⁻, blu⁺*, lambda-CI857 | r | Lambda-cI857 *blu⁺* transductants of SA194, -195, and -197; S. Adhya |

[a] K-10 and its derivatives are lysogenic for lambda.
[b] Sensitivity (s) or resistance (r) to streptomycin.
[c] Derived from *xyl⁺ str-r* recombinants in a mating of DF40 and X33.
[d] Derived from *arg⁺ str-r* recombinants in a mating of DF40 and X7099.

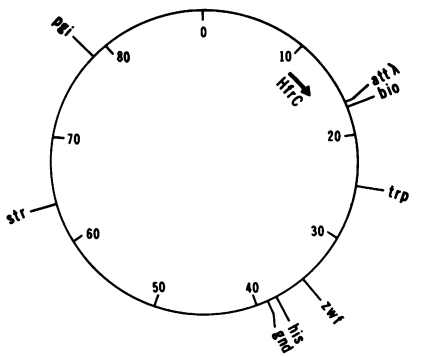


FIG. 3. *Genetic map of E. coli, adapted from Taylor and Trotter (12) showing origin of HfrC and positions of some of the genes mentioned.*

## RESULTS

**Isolation of phosphogluconolactonase mutants.** The isolation from phosphoglucose isomerase (*pgi*) mutants of double mutants also lacking glucose-6-phosphate dehydrogenase (*zwf*) has been described (5). The rationale for this was that since phosphoglucose isomerase mutants grew slowly on glucose by using the hexose monophosphate shunt exclusively (7), secondary mutants also lacking an enzyme of the shunt should be unable to grow at all on glucose. The double mutants cited were isolated in a procedure employing ethyl methane sulfonate mutagenesis of strain DF40 (*pgi⁻, zwf⁺*), followed by a penicillin enrichment step to decrease the fraction of survivors which could grow on either glucose or glucose-6-phosphate. The *pgi⁻, zwf⁻* double mutants were recognized by their failure to grow at all on glucose, while growing normally on gluconate and other carbon sources. In the same experiment two isolates of different phenotype were also recovered; they still grew on glucose, but far slower than DF40, and they contained normal levels of glucose-6-phosphate dehydrogenase and gluconate-6-phosphate dehydrogenase. A third such isolate, DF2023, was recovered in another experiment, which also mainly yielded *pgi⁻, zwf⁻* mutants from DF40; in that experiment ultraviolet mutagenesis was followed by intermediate growth in glycerol minimal medium and the penicillin selection was done in glucose minimal medium. These three mutants have now been found to lack 6-phosphogluconolactonase activity (Table 2).

We propose *pgl* as the genetic abbreviation for phosphogluconolactonase, and assign allele numbers *pgl-1*, *pgl-2*, and *pgl-3* to the lactonase mutations of strains DF1311, -1312, and -2023, respectively.

**Growth of the mutants.** Table 3 shows the growth of the *pgi⁻, pgl⁻* double mutants, their parent strain (DF40), and of a *pgi⁻, zwf⁻* strain (DF2000). Both in liquid culture and on plates, *pgi⁻, pgl⁻* strains grew much slower on glucose

than their *pgi*$^-$, *pgl*$^+$ parent. Indeed, with the usual 1- or 2-day incubation such strains would be scored as glucose-negative. However, upon 5 days of incubation they formed small colonies (which were not revertants), which, although small, were easily distinguishable from the "absolute" glucose negativity of a *pgi*$^-$, *zwf*$^-$ strain (Table 3). [Even the latter strain formed miniscule colonies on glucose, possibly because of leakiness in one of the blocks (6).]

The *pgl*$^-$ mutations were all selected in strains lacking phosphoglucose isomerase, and therefore severely defective in glucose metabolism. It was of interest, therefore, to examine the effect of *pgl* mutation on growth of a strain containing phosphoglucose isomerase. Such strains were formed by transduction with P1 by using a lysate prepared on K-10 (*pgi*$^+$, *pgl*$^+$) to infect cultures of DF1311, -1312, and -2023. Two kinds of transductants could be selected on glucose minimal plates, appearing as discrete colonies on fine confluent growth of the recipients. After purification and assay they proved to be, as expected, *pgi*$^-$, *pgl*$^+$ (like DF40) and *pgi*$^+$, *pgl*$^-$. Table 3 includes data on the growth of one of the latter type of transductant, DF1311t; *pgi*$^+$, *pgl*$^-$ transductants of DF1312 and -2023 had similar characteristics. Such isolates grew in glucose liquid minimal medium only slightly slower than the wild type or a *zwf*$^-$ strain. On minimal glucose plates the differences were somewhat more pronounced. The difference in growth between *pgl*$^-$ and wild type strains is caused by the *pgl*$^-$ mutation (rather than another mutation occuring during mutagenesis of DF40), as it was possible to select by two sequential transductions (the first to *pgi*$^-$ *pgl*$^+$, the second to wild type), *pgi*$^+$ *pgl*$^+$ derivatives of *pgi*$^-$ *pgl*$^-$ strains: such derivatives grew as well as strain K-10 on glucose plates.

TABLE 2. *Isolation of phosphogluconolactonase mutants*

| Strain | Enzyme activity[a] | | |
|---|---|---|---|
| | Glucose-6-P dehydrogenase | Gluconate-6-P dehydrogenase | Phosphogluconolactonase |
| K-10 (wild type) | 192 | 216 | + |
| DF40 (mutant of K-10) | 204 | 241 | + |
| DF1311 (mutant of DF40) | 201 | 246 | − |
| DF1312 (mutant of DF40) | 206 | 191 | − |
| DF2023 (mutant of DF40) | 231 | 213 | − |

[a] Values expressed as nanomoles per minute per milligram of protein. Extracts were prepared from broth-grown cultures. The gluconolactonase assay was scored qualitatively (*see* text). DF40 and its three derivatives all lack phosphoglucose isomerase (not shown).

**Mapping of pgl.** Strains DF1311, -1312, and -2023 are all derived from K-10, a prototrophic *str-s* Hfr Cavalli. To locate the genetic map position of *pgl*, these *pgi*$^-$, *pgl*$^-$ strains were

TABLE 3. *Growth of strains in minimal medium*[a]

| Strain | Growth rates[b] in liquid medium | | | Growth[c] on solid medium | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Glucose | | | Gluconate | Glycerol |
| | Glucose | Gluconate | Glycerol | 24 hr | 48 hr | 120 hr | 24 hr | 48 hr |
| | *min* | *min* | *min* | *mm* | *mm* | *mm* | *mm* | *mm* |
| K-10 | 75 | 71 | 93 | 1.2 | —[d] | — | 1.2 | 1.2 |
| DF40 (*pgi*$^-$) | 152 | 87 | 164 | — | 0.3 | — | 1.0 | 1.0 |
| DF1311 (*pgi*$^-$, *pgl-1*) | 415 | 82 | 90 | — | — | 0.4 | 0.7 | 1.1 |
| DF1312 (*pgi*$^-$, *pgl-2*) | 234 | 66 | 85 | — | — | 0.6 | 0.7 | 1.2 |
| DF2023 (*pgi*$^-$, *pgl-3*) | — | — | — | — | — | 0.3 | — | 0.6 |
| DF2000 (*pgi*$^-$, *zwf*$^-$) | No growth | 79 | 100 | — | — | <0.1 | — | 0.4 |
| DF1311t[e] (*pgi*$^+$, *pgl-1*) | 94 | 69 | 97 | 0.4 | — | — | 1.0 | — |
| DF2001 (*pgi*$^+$, *zwf*$^-$) | 86 | 71 | 94 | 0.9 | — | — | 1.0 | 1.4 |

[a] Minimal medium 63 was used, supplemented with 1 μg of thiamine-hydrochloride per ml and the carbon source at 0.4 or 0.6%. Growth rates in liquid medium were determined at 37 C as previously (7). Plates also contained 2% agar. Broth cultures were diluted and spread so as to give about 50 colonies per plate; average colony size was estimated after the indicated times of incubation at 37 C.

[b] Doubling time.

[c] Colony size.

[d] Dashes mean not done or not recorded.

[e] Strain DF1311t is a *pgi*$^+$ transductant of DF1311 (*see* text).

mated with a set of polyauxotrophic *str-r* F⁻ strains which also carried *pgi*⁻ and *pgl*⁺. Recombinants for the various nutritional markers (selected on the appropriately supplemented minimal mannitol or gluconate plates containing streptomycin) could be scored for the inheritance of *pgl*⁻, since those which were *pgl*⁻ would grow much slower on glucose than the female parent. In initial experiments no such recombinants appeared; however, when the recipients were also lambda lysogens, *pgl*⁻ recombinants were recovered. For example, when DF1311 was crossed with two strains which differed only by lysogeny [DF402 and -402 (lambda)], 0/134 *trp*⁺ recombinants from the DF402 mating were *pgl*⁻, whereas with DF402 (lambda), 55/80 *trp*⁺ and 11/60 *his*⁺ recombinants were *pgl*⁻. Thus *pgl* is probably a late marker in the donors (and with nonlysogenic recipients *pgl*⁻ recombinants were lost by zygotic induction). This was confirmed by its cotransduction with *bioA*. With DF403 (lambda) and P1 grown on DF1311, 0/128 *his*⁺ and 20/100 *bio*⁺ transductants were *pgl*⁻. With the other *pgl*⁻ strains, DF1312 and -2023, the figures for cotransduction with *bio* were 12/100 and 15/100, respectively.

More exact positioning of the locus was possible because of the existence of strains carrying deletions in this part of the chromosome (1; S. Adhya, *personal communication*; K. Manly and E. Signer, *personal communication*; J. Shapiro, *personal communication*). Adhya et al. (1) described the mapping of several genes in this region through the use of spontaneous chlorate-resistant mutants. Dr. S. Adhya and M. Schwartz (*personal communication*) have now further characterized a series of such mutants by also assaying them for the blue phenotype; blue mutants, when grown on a medium containing maltose, turn blue after iodine treatment. On the basis of their results, Adhya and Schwartz place a gene responsible for this phenotype, *"blu"*, between *chlD* and *lambda*. This is shown in Fig. 4, which also gives the presumed extent of deletions in three of their strains. We assayed 6-phosphogluconolactonase in these and other strains of this series, and found that all such strains showing the blue phenotype lacked 6-phosphogluconolactonase, whereas those which were not blue had the enzyme. These results are included in Fig. 4 for three strains; the open bar (SA315) indicates that this strain had the enzyme activity, and the closed bars (SA311 and -320) indicate that no activity was found. Thus *pgl* must also lie between *chlD* and *lambda*.

Indeed, the following results show that *"blu"* and *pgl* are probably identical. (i) Adhya and Schwartz have selected point *"blu"* mutants in this position. The three we have assayed, SA194, -195, and -197, lacked phosphogluconolactonase, whereas their non-blue parent, SA500, had it. (ii) Bacteriophage lambda has been found to transduce this *"blu"* locus; we found that non-*"blu"* transductants (SA515, -516, and -517) of the three point *"blu"* mutants had phosphogluconolactonase. (iii) *Pgi*⁺, *pgl*⁻ transductants of DF1311, -1312, and -2023 had the "maltose-blue" phenotype; the parent strains DF40 (*pgi*⁻ *pgl*⁺) and K-10 (*pgi*⁺ *pgl*⁺) did not. And, like the *"blu"* mutants mapping between *chlD* and *lambda*, the *pgi*⁺, *pgl*⁻ transductants contained blue-staining material when grown on maltose, but not when grown on glucose, galactose, mannitol, gluconate, or glycerol.

Adhya and Schwartz have found (*personal communication*) that when the maltose-blue phenotype was directly selected for, several classes of mutants appeared, only one of which mapped between *chlD* and *lambda*. Other classes consisted of mutants lacking enzymes of maltodextrin metabolism (Fig. 5), i.e., either maltodextrin phosphorylase or phosphoglucomutase, and mapped elsewhere. They therefore assume

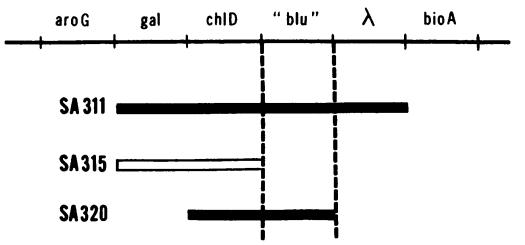


FIG. 4. *Pgl assay in some chlD-deletion strains. Lengths of bars show known loss of gene function (Adhya and Schwartz, in preparation). Open bars are for strains with phosphogluconolactonase, and closed bars for strains without.*

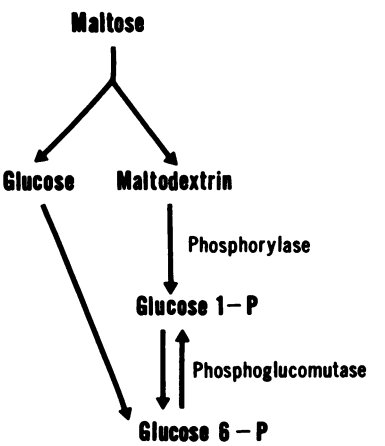


FIG. 5. *Maltose metabolism in E. coli (from M. Schwartz, 11).*

that the maltose-blue phenotype is likely to reflect the accumulation of maltodextrins from maltose. It is not obvious how this could also be caused by phosphogluconolactonase deficiency. We have tested strains blocked in other steps of the hexose monophosphate shunt, and have found that strains lacking glucose-6-phosphate dehydrogenase (e.g., DF2001), gluconate-6-phosphate dehydrogenase (e.g., DF1071), or gluconate-6-phosphate dehydrase (e.g., DF1070) did not present the maltose-blue phenotype. We have also constructed double mutants of genotype *pgl*$^-$, *zwf*$^-$, by crossing DF2001 (HfrC, *zwf*$^-$, *str-s*) with SA197 (F$^-$, *his*$^-$, *pgl*$^-$, *str-r*) and selecting *his*$^+$ recombinants; about half of these were also *zwf*$^-$. 7/7 *zwf*$^-$, *pgl*$^-$ recombinants did not give the blue phenotype, whereas 5/5 *zwf*$^+$, *pgl*$^-$ recombinants did. Thus, a *pgl*$^-$ strain must be *zwf*$^+$ to show the blue phenotype.

## DISCUSSION

It has previously been shown that *E. coli* mutants lacking one of the dehydrogenases of the hexose monophosphate shunt are not markedly affected in their growth on glucose. It is not surprising, then, that the new mutants described here, lacking 6-phosphogluconolactonase, another enzyme of the oxidative limb of the shunt, also grow well on glucose (a result confirming the earlier conclusion that this portion of the shunt is not essential). This is not to suggest, however, that cells lacking one of these enzymes are normal in all respects. Mutation in phosphogluconolactonase does have some effect on growth rate and, doubtless, on other cell functions. One such effect is the maltose-blue phenotype. It is not known how lactonase deficiency causes this. The fact that a lactonase mutant must contain glucose-6-phosphate dehydrogenase to show the effect suggests that this "*blu*" phenotype might be related to accumulation of 6-phosphogluconolactone. It would be interesting to test the effect in vitro of this compound on the enzymes of maltodextrin metabolism. We cannot yet speculate whether 6-phosphogluconolactone is involved in normal control of maltose metabolism, or of other pathways.

In contrast with the small effects on growth of the lactonase mutations in cells whose glucose metabolism is otherwise normal, when cells are also blocked in glycolysis because of lacking phosphoglucose isomerase, the oxidative pathway of the hexose monophosphate shunt becomes essential for growth on glucose, and the loss of one of its enzymes essentially prevents glucose growth entirely. Conventionally, any one of such double mutants [*pgi*$^-$, *zwf*$^-$; *pgi*$^-$, *gnd*$^-$ (*unpublished observations*); and *pgi*$^-$, *pgl*$^-$] would be scored as glucose-negative. However, it was the fact that the third class, *pgi*$^-$, *pgl*$^-$, was not quite as negative as the other two which allowed its recognition; such strains did grow very slowly on glucose. The most likely explanation for the leakiness of this class of mutants is not that the enzymatic deficiency might be incomplete (though this might be so for one of them, DF1312, which grows a little faster than the other two); rather, one might suppose that the spontaneous hydrolysis of the lactone, known to occur in vitro (*see* Fig. 2), also occurs in vivo. To show this, it would be useful to construct strains isogenic with DF1311 (*pgi*$^-$, *pgl*$^-$) but with deletions in *pgl*. This is in progress. It would be interesting to determine why, if spontaneous hydrolysis does occur, mass action does not allow as rapid hydrolysis of 6-phosphogluconolactone in the mutant as in the parent, and, therefore, equal growth on glucose.

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