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Genetic Control of the 2-Keto-3-Deoxy-D-Gluconate Metabolism in *Escherichia coli* K-12: *kdg* Regulon

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2-Keto-3-deoxy-gluconate (KDG), an intermediate of the hexuronate pathway in *Escherichia coli* K-12, is utilized as the sole carbon source only in strains derepressed for the specific KDG-uptake system. KDG is metabolized to pyruvate and glyceraldehyde-3-phosphate via the inducible enzymes KDG-kinase and 2-keto-3-deoxy-6-phosphate-gluconate (KDPG) aldolase. However, another inducible pathway, where the KDG is the branch point, has been demonstrated. Genetic studies of the KDG degradative pathway reported in this paper led to the location of KDG kinase-negative and pleiotropic constitutive mutations. The *kdgK* locus, presumably the structural gene of the kinase, occurs at min 69 and is co-transducible with *xyl*. The mutants, simultaneously constitutive for the uptake, kinase, and aldolase, define a *kdgR* locus at min 36 between the co-transducible markers *kdgA* and *oldD*. As to the nature of the control exerted by the *kdgR* product, we have shown the following. (i) Thermosensitive mutants of the *kdgR* locus are inducible at low temperature but derepressed at 42 C for the three operons—*kdgT* (transport system), *kdgK*, and *kdgA* (KDPG aldolase). (ii) The $kdgR^+$ allele is dominant to the *kdgR* constitutive allele. (iii) A deletion in *kdgA* extending into the regulatory gene, *kdgR*, leads to a constitutive expression of the nondeleted operons—*kdgT* and *kdgK*. These properties demonstrate that the *kdg* regulon is negatively controlled by the *kdgR* product. It is presumed that differences in operator and in promotor structures could explain the strong decoordination, respectively, in the induction and catabolic repression, of these three enzymes activities.

2-Keto-3-deoxy-gluconate (KDG) has been shown by Ashwell (29) to be the intermediate common to the degradation of D-glucuronate and D-galacturonate in *Escherichia coli* (Fig. 1). This aldonic acid is phosphorylated by a specific 2-keto-3-deoxy-gluconate kinase (KDG kinase, EC 2.7.1.45) (4, 18) and then enters the Entner-Doudoroff pathway where it is cleaved by 2-keto-3-deoxy-6-phosphate (P)-gluconate aldolase (KDPG aldolase, EC 4.1.2.14) to pyruvate and glyceraldehyde-3-P (15, 19). We have recently identified a specific 2-keto-3-deoxy-gluconate transport system which brings about the uptake of KDG into the cell against a concentration gradient (16, 20).

The structural genes specifying these three functions are under the control of a common regulatory gene and form the *kdg* regulon, as has been reported recently in a preliminary note (21).

Although the wild type carries a gene coding for the KDG transport system, it is unable to grow on this compound as the sole carbon and energy source. We explain this peculiarity by the observation that the KDG uptake system is strongly repressed and only weakly inducible in such an organism (22). However, we have shown that mutation in a regulatory component of the *kdg* regulon is sufficient to allow growth on KDG (20, 22). These mutations, which occur spontaneously with high frequency, derepress the gene coding for the KDG transport system (20). *E. coli* strains carrying such a mutation are able to metabolize exogenous KDG by means of the three contiguous steps—KDG uptake, KDG kinase, and KDPG aldolase.

Recent genetic analysis showed that the structural gene of the KDPG aldolase (*kdgA*) is located at about min 36 on the *E. coli* map (6, 7, 23, 24, 31) and a structural gene (*kdgT*) of the KDG uptake system has its operator locus (*kdgP*) at min 77.5 (20, 31).

In this paper, the chromosomal location of the regulatory gene (*kdgR*) and of the presumptive

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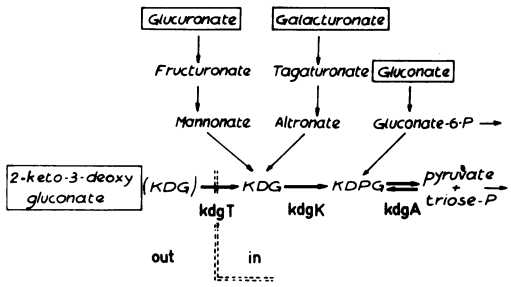

FIG. 1. *Degradative pathways of 2-keto-3-deoxy-D-gluconate, hexuronate, and gluconate in E. coli K-12.*

structural gene for the KDG kinase (*kdgK*) will be reported. Furthermore, the previously suggested hypothesis of a negative control exerted by the *kdgR* product (22) will be strengthened here by studies of: (i) the dominance effects of the *kdgR* alleles, (ii) the physiological aspects of thermosensitive regulatory mutants, and (iii) deletion of the regulator gene.

MATERIALS AND METHODS

Growth of bacteria. Bacteria were grown in liquid or on solid media as reported previously (22, 23).

Bacterial strains. All the bacterial strains utilized were derivatives of *E. coli* K-12. The mutants of the KDG degradative pathway were obtained from the Hfr strain P4X (auxotrophic for the methionine), or from derivatives of it. The different genetic markers of these strains are listed in the Table 1. Besides the genetic symbols employed by Taylor (31), we have introduced the symbols *kdgA* (structural gene of the 2-keto-3-deoxy-6-P-gluconate aldolase), *kdgK* (locus of the 2-keto-3-deoxy-glucono-kinase-negative mutations), *kdgT* (structural gene of a component of the KDG transport system), *kdgP* (operator site of the *kdgT* operon), and *kdgR* (regulatory gene common to the three *kdgT*, *kdgK*, and *kdgA* operons). Figure 2 shows the location of these loci and of the relevant markers used.

Enzyme assays. Enzymatic activities were determined on crude extracts obtained from sonically disrupted bacteria or from bacteria treated with toluene (22). The details of the KDG kinase, KDPG aldolase and KDG transport system have been described elsewhere (18, 19, 16). The 6-P-glucose dehydrogenase was assayed under the same conditions as those described previously (22). The specific activities are expressed in nanomoles of substrate transformed per minute per milligram dry weight.

Genetic techniques. The methods used for transduction with the phage P1*kc* and for conjugation with Hfr strains were the same as those reported elsewhere (23).

Two methods for the isolation of KDG kinaseless mutants (*kdgK* mutation) have been employed. In the first one, the Hfr strain P4X was treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (400 μg/ml) (1) for 120 min at 37 C. The bacteria were then suspended in rich medium and grown overnight. After the bacteria were plated on nutrient agar, resulting colonies were replicated on both hexuronates and glycerol minimal medium. Mutants impaired only in their growth on both hexuronates were selected. Strain KO1 was isolated by this method.

The second method is based on the sensitivity of the strain A 314 (KDPG aldolase negative) to the compounds that generate KDPG. The accumulation of this compound led to a strong growth inhibition on glycolytic and gluconeogenic substrates (19, 25). Thus, secondary mutants that eliminate the toxic effect of the KDPG formation by a block preceding the aldolase step can be selected easily. The strain CA1 (*kdgR*, *kdgA*), a derivative of strain A 314, is unable to grow on glycerol minimal medium supplemented by either one of the hexuronates or KDG (20). So, when this strain is plated on a mixture of galacturonate (1 mg/ml), KDG (1 mg/ml), and glycerol (2 mg/ml), KDG kinase-negative mutants are specifically selected. We will see later why a minimal medium with an hexuronate as the sole carbon source also allows the specific selection of KDG kinase-negative mutants from A 314 or one of its derivative strains.

The selection of regulatory mutants (*kdgR* and *kdgR* [ts] mutations) was based upon the observation, reported elsewhere, that exogenous KDG, which is unable to induce its transport system (20, 22), cannot support the growth of the wild-type strain. Therefore, it behaves as a noninducing substrate towards its uptake system. A minimal medium with KDG (1 or 2 mg/ml) as the sole carbon source is a selection medium for KDG uptake constitutive mutants. Two classes of such constitutive mutants have been characterized among the clones arising spontaneously on this medium: *kdgP* (operator constitutive mutants of the *kdgT* operon [20]); *kdgR* (mutants simultaneously derepressed for the three enzymatic activities of the *kdg* regulon [22]).

Independent cultures in 1 ml of nutrient broth from strain S 39 were streaked on a KDG minimal medium. After 48 h at 37 C, a Kdg^+ clone was picked from each streak, purified, and analyzed by a qualitative colorimetric assay for the constitutivity of the kinase and aldolase (22). The strains CS 391, 394, and 395 were selected by this procedure.

The *kdgR* (ts) mutants were isolated in the following way. Independent nutrient broth cultures of P4X were spread at a suitable dilution (to give 50 to 200 clones by plate) on KDG (2 mg/ml) minimal medium and incubated for 48 h at 42 C. The plates were then replicated onto KDG and onto galacturonate minimal medium. After 24 h at 28 C, the replicated clones of phenotype Kdg^- but galacturonate positive at 28 C were picked and purified. The qualitative colorimetric assay (22) showed that all these mutants thermosensitive for growth on KDG were constitutive for the kinase and aldolase when grown on nutrient broth at 42 C. They belong, therefore, to the *kdgR* constitutive class at high temperature.

Chemicals. [^{12}C]KDG potassium salt used for the enzymatic assay and bacterial growth was synthetized enzymatically from the D-altronic acid (26). [1-^{14}C]- and [U-^{14}C]KDG were prepared with resting

TABLE 1. *Bacterial strains*

Strains	Sex	Chromosomal genotype	Derivation
P4X	Hfr	*metB, thi*	E. Wollman
PA 309	F⁻	*thi, thr, leu, arg(HE), his trp, lac, gal, malA, xyl, mtl, str*	E. Wollman
AB 313	Hfr	*thr, leu, met, thi, str*	C. Babinet
P10-dct 1[a]	Hfr	*thr, leu, dct-1, kdgK*[a]	W. W. Kay (14)
A 314	Hfr	*metB, thi, kdgA2*	P4X, J. Pouysségur (23)
AD 3141	Hfr	*metB, thi, kdgA2 edd-21*	A 314, J. Pouysségur and F. Stoeber (19)
MAD 40	F⁻	*his-1, man-1, kdgA2, edd-21, gal-3 str*	AD 3141 × K 63, J. Pouysségur (23)
K 63	F⁻	*his-1, oldD88, man-1, gal-3, str*	P. Overath
AK 3141	Hfr	*metB, thi, kdgA2, kdgK2*	Spontaneous mutant of A 314 (this paper)
K 3141	Hfr	*metB, thi, kdgK2*	*kdgA*⁺ transductant from AK 3141
K 01	Hfr	*metB, thi, kdgK1*	NTG[b] mutant of P4X (this paper)
K 85	F⁻	*arg(HE), his, trp, lac, gal, mtl, xyl, kdgK2, str*	K 3141 × PA 309 (this paper)
CA 1	Hfr	*metB, thi, kdgR1, kdgA2*	CS 391 × P1 (A 314)
CAK 101	Hfr	*metB, thi, kdgR1, kdgA2, kdgK10*	Spontaneous mutant of CA1, J. Pouysségur and A. Lagarde (20)
CS 391	Hfr	*metB, thi, oldD88, kdgR1*	Spontaneous mutant of S 39
CS 394	Hfr	*metB, thi, oldD88, kdgR2*	
CS 395	Hfr	*metB, thi, oldD88, kdgR3*	
S 39	Hfr	*metB, thi, oldD88*	P4X × P1 (K 63), J. Pouysségur (23)
C 31	Hfr	*metB, thi, kdgR10*	Spontaneous mutant of P4X (this paper)
C 41	Hfr	*metB, thi, kdgR11*	
C 51	Hfr	*metB, thi, kdgR12*	
C 81	Hfr	*metB, thi, kdgR13*	
C 91	Hfr	*metB, thi, kdgR14*	
C 122	Hfr	*metB, thi, kdgR15*	
C 126	Hfr	*metB, thi, kdgR16*	
C 131	Hfr	*metB, thi, kdgR17*	
C 141	Hfr	*metB, thi, kdgR18*	
DZ 47	F⁻	*his, pgi, str* Δ *(zwf, edd, kdgA, kdgR)*	Fraenkel and S. Banerjee (8) and this paper

[a] The strain P10-dct1 carries a further mutation *kdgK* caused by a deletion of *dct* extending into *kdgK* (see Results).

[b] NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

cells of a KDG kinaseless *E. coli* strain from [6-^{14}C]- and [U-^{14}C]glucuronate, respectively (27). [^{14}C]glucuronate was purchased from the Radiochemical Centre, Amersham, England.

RESULTS

Evidence for a secondary pathway metabolizing KDG. Some of the physiological properties of the KDG kinaseless mutants (*kdgK*) and the induction mechanism of the kinase and aldolase have been described (22). Besides the loss of the KDG kinase activity, large amounts of KDG were excreted in the medium when these mutants were grown on glycerol supplemented with either glucuronate or galacturonate (27). These observations support the hexuronate metabolic pathway stated by Ashwell (2), although some others also indicate a secondary pathway.

More than 60 hexuronate-negative mutants were induced with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and selected after penicillin enrichment (5) on minimal medium containing both hexuronates. No kinaseless mutant was found by this selection. Since we suspected the existence of two kinase activities in the wild type, we omitted the penicillin step in the subsequent isolation of kinaseless mutants (see above). Strain KO1 was thus selected. This mutant, which shows a 98% reduction of the kinase activity (22), is able to grow at a low rate on either glucuronate or galacturonate. All the independent kinaseless mutants isolated spontaneously by resistance to KDPG toxicity show the same residual growth on hexuronate. Two hypotheses could explain these observations.

(i) There may exist a second KDG kinase in the wild type. This activity is not revealed by

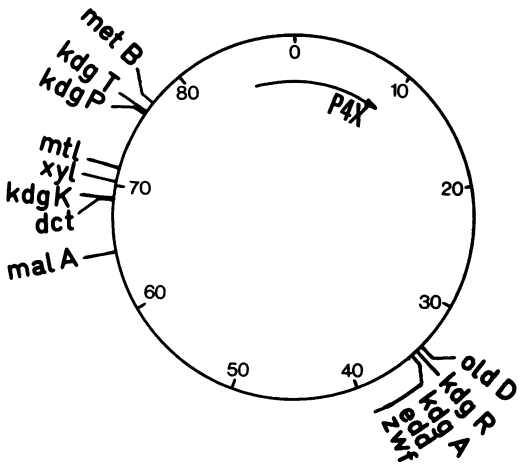

FIG. 2. *Simplified chromosomal map of E. coli K-12 according to Taylor and Trotter (31).*

our normal assay because the optimal conditions for its activity are different from those of the missing KDG kinase.

(ii) KDG could be metabolized by another metabolic pathway.

The existence of two KDG kinase activities analogous to the two gluconate kinases of *E. coli* (A. Hung, A. Orozco, and N. Zwaig, Bacteriol. Proc., p. 148, 1970), seems very unlikely since a double mutant (kinase negative, aldolase negative) such as strain AK 3141 grows on both hexuronates as well as its aldolase-positive derivative, strain K 3141.

Further observations agree with the hypothesis that a double mutant (kinase negative, aldolase negative) which carries a *kdgR* constitutive mutation, thus derepressing the KDG transport system (strain CAK 101), is able to grow at low rate on KDG in spite of the double block (kinaseless, aldolaseless). Since the two dehydrases generating KDG from the aldonic acids are irreversible (Fig. 1) (29), the residual growth of strain CAK 101 on KDG or hexuronate supports the idea that KDG itself is metabolized by another pathway. The generation time of strain CAK 101 on galacturonate or KDG is about 3 to 4 h (Fig. 3). On glucuronate the generation time is greater; the reason for this is unknown. A long lag on KDG is observed when CAK 101 is pregrown on glycerol, but this lag does not occur when CAK 101 is pregrown on glycerol plus hexuronate (Fig. 3). Therefore, this secondary pathway seems to be inducible. This property has been pointed out by Lagarde et al. in their paper on the kinetics of KDG uptake (16).

Moreover, when strain A 314 (*kdgA*) is spread on minimal medium with either glucuronate or galacturonate as the sole carbon source, some colonies arise after 3 days at 37 C. These secondary mutants are easily distinguished from the *kdgA*$^+$ revertants appearing in 2 days at a 10^2-times lower frequency. The analysis of 40 such colonies isolated on each hexuronate has shown that all these mutants carry a kinase-negative mutation as a secondary defect. This event prevents the accumulation of the toxic compound in the cell and reveals the new metabolic pathway of hexuronates which is masked in the *kdgA* strain by the KDPG toxicity.

Mapping of the kdgK$^-$ mutations. Although kinase-negative mutants are still able to grow on hexuronates at low rate (as mentioned above), the galacturonate or glucuronate phenotype can still be used to distinguish the *kdgK*$^+$ allele from the *kdgK*$^-$ on solid medium.

A preliminary cross between strain KO1, a derivative of strain Hfr P4X, and the F$^-$ strain PA 309, showed the following percent inheritance of the *kdgK*$^-$ allele: Thr$^+$ Leu$^+$ (*str*), 1%; Arg$^+$ (*str*), 0%; Xyl$^+$ (*str*), 60%; Mal$^+$ (*str*), 15%; Mal$^+$ (*metB*$^+$), 23%; and His$^+$ (*metB*$^+$), 16%. This result suggests a position for the *kdgK1* mutation in the *xyl* region. Among the *malA*$^+$ recombinants, the *kdgK1* allele is inherited at higher frequency than the *xyl*$^+$ character. So these observations support the order *malA-kdgK-xyl* (Table 2). A comparable mating with mutant K 3141 carrying an independent kinase-negative mutation has given us similar results (Table 2). In the three-point cross shown in the Table 2, the lowest recombinant

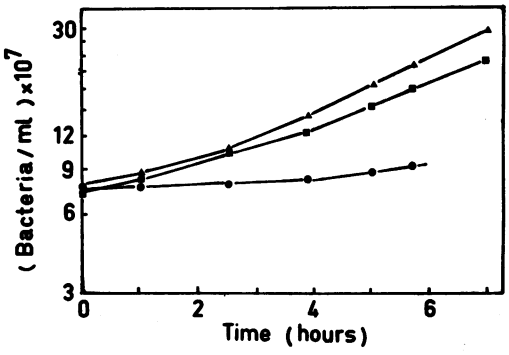

FIG. 3. *Growth of the strain CAK 101 on KDG and galacturonate by the secondary pathway. Cells of the strain CAK 101, grown on glycerol (3 mg/ml) minimal medium, were washed and suspended in KDG (2 mg/ml) minimal medium (●). Cells of the strain CAK 101 grown on glycerol plus galacturonate minimal medium were washed and suspended into KDG (2 mg/ml) minimal medium (■) and galacturonate (2 mg/ml) minimal medium (▲).*

class is *malA*, *kdgK*, *xyl*, which suggests the order *malA-kdgK-xyl*. Furthermore, from the size of the two classes (*kdgK*$^+$, *xyl*$^-$) and (*kdgK*$^-$, *xyl*$^-$), it follows that the *kdgK* marker is closer to *xyl* than to *malA*. This is strengthened by the three-point cross from the mating between the recombinant K 85 (*kdgK2*) and the Hfr AB 313. The analysis presented in Table 3 indicates the order *kdgK-xyl-mtl*. The distance *kdgK-xyl* appears to be the same as *xyl-mtl*, namely about 1 min (31).

The results of co-transduction between *xyl* and the *kdgK1* and *kdgK2* mutations are summarized in Table 4. The facts that *kdgK* is co-transducible with *xyl* but not with *mtl* and that the co-transduction frequencies of *xyl-kdgK* and *xyl-mtl* are equal (2 to 6%) demonstrate at once that the order of the three markers is *kdgK-xyl-mtl* and that *kdgK* is located at about min 69 on the *E. coli* chromosomal map (31).

In the same area, min 69, mutations affecting the uptake of the dicarboxylic acid transport (locus *dctA*) have been mapped (8, 14). So to locate *kdgK* more precisely, we attempted to co-transduce *kdgK* and *dct* with the P10 dct-1 strain and several kinase-negative mutants (*dct*$^+$). These experiments were unsuccessful. A further analysis of the strain P10-*dct-1* has shown us that it carries a *kdgK*$^-$ mutation. In the last part of Table 4, it can be seen that the frequency of co-transduction of *xyl* with either *dct* or *kdgK* is the same. Not only is the frequency of co-transduction of *kdgK* with *xyl*

TABLE 2. *Conjugation—study of the transmission of the kdgK*$^-$ *allele and order of the markers malA, kdgK, and xyl*[a]

Donor	Recipient	Selected markers	No. analyzed	Inheritance of unselected markers (%)				
				argEH$^+$	*xyl*$^+$	*malA*$^+$	*kdgK*$^-$	
K 01 (*metB*, *kdgK1*, *str*)	PA 309 (*argEH*, *xyl*, *malA*)	*xyl*$^+$ (*str*)[a]	89	100	—	19	60	
		malA$^+$ (*str*)	71	6	13	—	15	
		malA$^+$ (*metB*$^+$)	73	0	11	—	23	
				argEH$^+$	*mtl*$^+$	*xyl*$^+$	*malA*$^+$	*kdgK*$^-$
K 3141 (*metB*, *kdgK2*, *str*)	PA 309	*xyl*$^+$ (*str*)[b]	269	41	77	—	16	60
		malA$^+$ (*str*)	202	25	34	41.5	—	43.5

Analysis of the three-point cross (*malA*, *kdgK*, *xyl*) from the *malA*$^+$ (*str*) recombinants:

(*kdgK*$^-$ *xyl*$^+$)	(*kdgK*$^-$ *xyl*$^-$)	(*kdgK*$^+$ *xyl*$^+$)	(*kdgK*$^+$ *xyl*$^-$)
37	6	3.5	53.5

[a] The Xyl$^+$ recombinants were selected on EMB nutrient broth containing 10 mg of xylose per ml.

[b] In this case, the Xyl$^+$ recombinants were selected on minimal medium with xylose at 2 mg/ml. The artifact of selection, 100% *arg*$^+$, in *a* is not well understood. It seems that the *xyl*$^+$ *arg*$^-$ recombinants are unable to give a fermentative reaction in the EMB medium. This artifact is eliminated selecting the recombinants on minimal medium, *b*.

TABLE 3. *Conjugation—order of the markers kdgK, xyl, mtl*[a]

Donor	Recipient	Selected marker	No. analyzed	Inheritance of unselected markers (%)		
				kdgK$^+$	*xyl*$^+$	*mtl*$^+$
AB 313	K 85 (*kdgK2*, *xyl*, *mtl*)	*xyl*$^+$	75	76	—	88
		mtl$^+$	128	51	65.5	—
		kdgK$^+$	364	—	86	75

Analysis of the three-point cross from the *kdgK*$^+$ recombinants:

(*xyl*$^+$ *mtl*$^+$)	(*xyl*$^+$ *mtl*$^-$)	(*xyl*$^-$ *mtl*$^+$)	(*xyl*$^-$ *mtl*$^-$)
73	13.5	2.5	11

[a] The Hfr AB 313 was counter-selected with the late markers *thr*, *leu*, and *met* of this strain. The selection of the three kinds of recombinants was carried out on minimal medium with either xylose (2 mg/ml), mannitol (2 mg/ml), or galacturonate (2 mg/ml). The recombinants were purified once on the same medium and analyzed by replica plating on the other media.

TABLE 4. *Co-transduction study of the xyl and kdgK markers*

Donor	Recipient	Selected marker	No. analyzed	Inheritance of the unselected markers (%)			
PA 309 (*xyl, mtl*)	K01 (*kdgK1*)	*kdgK*$^+$	327	*kdgK*$^+$ —	*xyl*$^-$ 6.5	*mtl*$^-$ <0.3	
K 01 (*kdgK1*)	PA 309 (*xyl, mtl*)	*xyl*$^+$	162	*kdgK*$^-$ 1.5[a]	*xyl*$^+$ —	*mtl*$^+$ 4	
PA 309 (*xyl, mtl*)	K 3141 (*kdgK2*)	*kdgK*$^+$	213	*kdgK*$^+$ —	*xyl*$^-$ 4	*mtl*$^-$ <0.5	
P10-dct1 (*dct, kdgK*)	PA 309	*xyl*$^+$	170	*kdgK*$^-$ 29	*dct*$^-$ 29	*mtl*$^+$ 15	*xyl*$^+$ —

[a] Among the Xyl$^+$ transductants, two inherited the galacturonate ± phenotype of the *kdgK*$^-$ allele. These two transductants show no kinase activity and, moreover, excrete KDG into the culture medium when supplied with an hexuronate. These properties indicate the presence of the *kdgK*$^-$ allele in the two Xyl$^+$ transductants.

increased (from 5 to 30%), but also no segregation of the *xyl* and *dct* markers is observed. These results and the low frequency of reversion of the strain P10 from *dct-1* to *dct*$^+$ or *kdgK*$^+$ ($<10^{-9}$) strongly suggest the presence of a deletion covering *dct* and *kdgK*. The end of this deletion is at a point that is 30% co-transductible with *xyl*, but we do not know whether *dct* is in or outside the *kdgK-xyl* segment. On the other hand, some of the KDG kinaseless mutants that we have isolated are simultaneously impaired in their growth on dicarboxylic acids. The selection of such deletions covering *kdgK* and *dct* at a high frequency bear out the mapping of these two loci in the same area (min 69).

Physiological aspects of the kdgR constitutive mutants. We have seen elsewhere (22) that KDG, although the presumptive true inducer of the *kdg* regulon, behaves as a noninducing substrate. This property has been used for selecting strains constitutive for the KDG transport system. Besides the *kdgP* mutants affected in the operator of the *kdgT* operon (20), other independent mutants (*kdgR*) have been isolated. The mutants of the latter class show a pleiotropic derepression of the three operons *kdgT*, *kdgK*, and *kdgA* (21, 22). The isolation of such pleiotropic mutants, the expression of which depends on the growth temperature, has provided information about the nature of the control exerted by the *kdgR* product.

The three *kdgR* strains (CS 391, 394, 395) which are constitutive for KDG uptake, kinase, and aldolase (22) are able to grow as well at 28 C as at 42 C with KDG as the sole carbon source. The nine independent mutants, C 31 to C 141, either grow very slowly or not at all on KDG at 28 C, whereas their growth on this compound is normal at 42 C. We have reported the specific activities of kinase and aldolase and the growth on KDG of these mutants at both temperatures (Table 5). When they were grown without inducer at high temperature, the levels of kinase and aldolase were, respectively, 10 to 12 times and 5 to 6 times higher than at low temperature. The KDG transport system was not assayed in these mutants, but at low temperature the growth on KDG reflects directly the level of this activity, since KDG induces kinase and aldolase. It is noteworthy (Table 5) that the mutants showing the lower kinase activity at 28 C (C 91, C 126, C 131) are totally negative for the growth on KDG at low temperature. These mutants are strongly repressed at low temperature, like the parental strain. That these thermosensitive mutations affect the control of the *kdg* regulon specifically is shown by the fact that the 6-phosphoglucose dehydrogenase activity is not modified by the growth temperature (Table 5). Moreover, at low temperature, these nine mutants are inducible for the kinase and aldolase by the hexuronates (Table 6). Therefore, at 28 C the control of the biosynthesis of these enzymes is qualitatively not changed by the *kdgR* (ts) mutations.

A further study of one of these mutants (strain C 91, grown on glycerol minimal medium at different temperatures) is shown in Fig. 4. The three enzymes of the *kdg* regulon, repressed at low temperature, are simultaneously derepressed between 32 and 35 C. Above this critical temperature, the *kdg* regulon is no longer controlled. We will see below how these results are consistent with a negative control exerted by the *kdgR* product.

Mapping of the kdgR locus. The difficulty of constructing the strain CA1 (*kdgA, kdgR*) by transduction suggested the presence of the *kdgR* locus in the *kdgA* region. This first indication was borne out by the cross between strain C 31 (Hfr P4X carrying a *kdgR* [ts] mutation) and the F$^-$ strain MAD 40 (*man, kdgR*$^+$, *edd, his*). Among the Man$^+$ (*str*) recombinants, the per-

TABLE 5. *Growth temperature effect on the kdgR thermosensitive mutants*[a]

Strain	Sp act at 28 C[b]			Growth on KDG at 28 C	Sp act at 42 C[b]			Growth on KDG at 42 C
	KDG kinase	KDPG aldolase	Glucose-6-P-dehydrogenase		KDG kinase	KDPG aldolase	Glucose-6-P-dehydrogenase	
C 31	315	700		+	1,000	1,360		++
C 41	165	465		±	1,420	1,530		++
C 51	135	335		±	1,180	1,310		++
C 81	165	355		±	1,250	1,435		++
C 91	100	330		−	1,090	1,455		++
C 122	160	410	80	+	1,200	2,040	105	++
C 126	100	265	80	−	1,240	1,900	100	++
C 131	125	475	80	−	1,115	1,760	90	++
C 141	170	380	85	+	1,260	1,810	100	++
P 4X	30	210	85	−	75	320	90	−

[a] The strains were grown aerobically on glycerol (3 mg/ml) minimal medium at 28 and at 42 C. The cells were harvested in the exponential phase at a bacterial density of 6.10^8/ml. The specific activities measured from toluene-treated cells (22) are the average of two independent experiments. The growth of patches on KDG replicated from nutrient agar to KDG (2 mg/ml) was scored after 24 h of incubation at either 28 or 42 C. Strains which showed no growth at 28 C after 24 h of incubation were still negative after 3 to 4 days of incubation.

TABLE 6. *Induction of the kdgR thermosensitive mutants at 28 C*[a]

Strains	Sp act	
	KDG kinase	KDPG aldolase
C 31	510	630
C 41	505	655
C 51	525	575
C 81	640	620
C 91	495	520
C 122	545	610
C 126	475	610
C 131	380	515
C 141	420	470
P4X (wild type)	365	610

[a] The strains were grown aerobically at 28 C on minimal medium with glycerol (2 mg/ml) as energy and carbon source, and galacturonate (2 mg/ml) as a substrate inducer. The values are the average of duplicate experiments. The activities were determined as stated in Table 5.

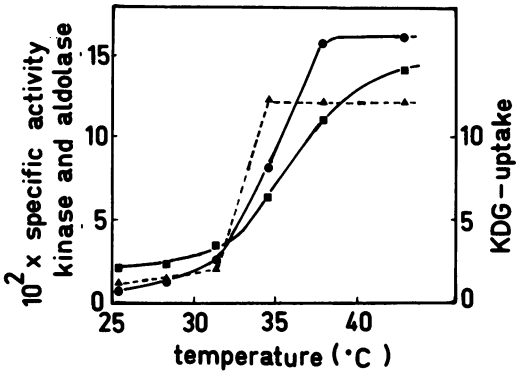

FIG. 4. *Specific activities of the enzymes of the kdg regulon as a function of the growth temperature for the kdgR (ts) mutant, C 91. Strain C 91 was grown on glycerol (3 mg/ml) minimal medium at different temperatures. The three activities of KDG uptake (▲), KDG kinase (●), and KDPG aldolase (■) were assayed on cells harvested on the late logarithmic phase. Each point is the average of duplicate experiments.*

centage of co-transmission of the Hfr markers are: *kdgR* (ts), 68%; *kdgA*$^+$, 68%; *edd*$^+$, 67%; and *his*$^+$, 54%. The order has been established by the results of transduction presented in Table 7.

Subsequent to our report that the two loci, *kdgA* and *oldD*, are co-transducible (23), we isolated *kdgR* mutants in an *oldD*$^-$ strain.

Table 7 shows that the three independent *kdgR* mutations are co-transducible with both *kdgA* and *oldD*. In each case, the smallest class resulting from the low frequency of quadruple crossing-over is *kdgR*$^-$–*kdgA*$^-$ (experiment 1, Table 7). This result is consistent with the order *kdgA*, *kdgR*, *oldD*. Whereas the frequencies of co-transduction between *oldD* and the three independent *kdgR* mutations vary from 0.55 to 0.78, the ratio of the distances *kdgR–oldD*/*kdgR–kdgA*, calculated from the frequencies of crossing-over, is constant (Fig. 5). This conclusion is reached from the analysis of the segregation of the *kdgR* marker among the *oldD*$^+$, *kdgA*$^-$ transductants (experiment 1a, 3, 4 in Table 7) or among the *kdgA*$^+$, *oldD*$^-$ transductants (experiment 2 in Table 7). Therefore, the three constitutive mutations appear closely linked and we suggest that they belong to the same locus.

TABLE 7. *Transduction of the kdgR locus and order of the markers kdgA, kdgR, and oldD*[a]

Expt	Donor	Recipient	Selected markers	No.	Inheritance of unselected markers (%)			
					edd⁻	*kdgA*⁻	*kdgR*⁺	*oldD*⁺
1a	AD 3141 (*kdgA*, *edd*)	CS 391 (*kdgR*, *oldD*)	*oldD*⁺	452	42	42	78	—
1b			*oldD*⁺ *edd*⁻	303	—	99.5	100	—
	Analysis of the three-point cross from the *oldD*⁺ transductants: (*kdgR*⁻ *kdgA*⁺) 22 (*kdgR*⁻ *kdgA*⁻) 0 (*kdgR*⁺ *kdgA*⁺) 36 (*kdgR*⁺ *kdgA*⁻) 42							
					edd⁺	*kdgA*⁺	*kdgR*⁻	*oldD*⁻
2a	CS 391	AD 3141	*kdgA*⁺	279	88	—	46.5	11
2b			*kdgA*⁺ *kdgR*⁻	96	99	—	—	30
					edd⁻	*kdgA*⁻	*kdgR*⁺	*oldD*⁻
3	AD 3141	CS 394 (*kdgR*, *oldD*)	*oldD*⁺	152	27	28	55	—
4	AD 3141	CS 395 (*kdgR*, *oldD*)	*oldD*⁺	152	33	33	62	—

[a] The *edd*⁻, *kdgA*⁻, *oldD*⁻ characters were analyzed as recently reported (23). The *kdgR*⁻ allele is distinguished from the wild-type allele *kdgR*⁺ by growth on KDG after 48 h. When the presence of the *kdgA* allele precludes the use of this phenotype, the *kdgR*⁺ and *kdgR*⁻ alleles were distinguished by growth on glycerol (2 mg/ml) plus KDG (1 mg/ml). *kdgR*⁻ strains (derepressed for the KDG permease) are inhibited on this medium by the accumulation of the toxic KDPG.

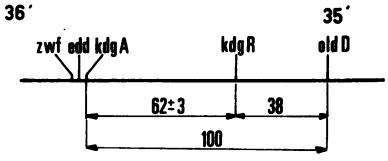

FIG. 5. *Location of kdgR between the markers kdgA and oldD, co-transducible at a frequency of 0.15 (23). The distances relative to 100 (oldD-kdgA) have been calculated as the inverse of the crossing-over frequencies derived from the segregation of kdgR between the oldD and kdgA markers (data derived from Table 7). The "error" is the variation found with the three independant mutations. The order of the clustered markers zwf, edd, kdgA has recently been established by Fraenkel and Banerjee (8).*

As to the thermosensitive constitutive mutation, we have shown that at least five of them are co-transducible with *kdgA* (Table 8). To locate these mutations more precisely, we have transduced a *kdgR* (ts) strain with phage P1 grown on a *kdgR* mutant (last line of the Table 8). The fact that there is no segregation of the *kdgR*⁺ wild-type allele among the *kdgA*⁺ transductants, i.e., *kdgR*⁻ (59%), *kdgR* (ts) (41%), suggests that the *kdgR* and *kdgR* (ts) mutations affect the same gene.

Dominance effect between kdgR constitutive and kdgR⁺ alleles. When the strain AD 3141 (*kdgA*⁻) was transduced with phage P1 grown on CS 391 strain (*kdgA*⁺, *kdgR*⁻), the ratio of the Kdg⁺ transductants (*kdgA*⁺, *kdgR*⁻) to the galacturonate-positive transductants (*kdgA*⁺) is lower than 0.1. But, in the same experiment, the frequency of the Kdg⁺ phenotype among the galacturonate-positive transductants is 0.46 (experiment 2a in Table 7). This observation indicates that KDG used as the sole carbon source does not allow the selection of all the (*kdgA*⁺-*kdgR*⁻) transductants. This negative interference, which is reproducible, can be explained if the *kdgR*⁺ allele is *trans* dominant to *kdgR*⁻. The *kdgR*⁺ product which is present in each cell could prevent the growth of some (*kdgR*⁻-*kdgA*⁺) transductants on KDG since it strongly represses the KDG uptake system. To check this interpretation we have studied the effect of phenotypic expression (for 10 generations in nutrient broth) on the ratio of Kdg⁺/galacturonate-positive transductants.

After this treatment the ratio was 0.45, which is the co-transduction frequency between *kdgA* and *kdgR* (0.46). The expression of the recombinant *kdgR* genotype can only occur after the dilution of the *kdgR*⁺ product in the transduced cells.

Using the same technique we have studied the second class of Kdg⁺ mutations (*kdgP*) which are co-transductible with *metB* (20). The ratio of the Kdg⁺ (*kdgP*⁻, *metB*⁺) to the Glu⁺ (*metB*⁺) transductants is 0.23 without expres-

sion of the transduced cells. This value is very similar to the co-transduction frequency of these two markers (0.26). In this case, the *kdgP*$^+$ allele does not exert a trans dominant effect on the *kdgP* constitutive allele. This is in agreement with the hypothesis that *kdgP* is the operator site of the *kdgT* operon (20).

Deletion of the kdgR locus. Fraenkel and Banerjee (8) have isolated a set of deletions to determine the order of the three clustered genes *kdgA*, *edd*, *zwf*. One of these deletions, strain DZ 47, shows an inhibition of growth on glycerol when KDG is added to the medium. This inhibition by KDG is a property of *kdgA*$^-$ mutants which are also derepressed for the KDG-uptake system (*kdgT* operon) (20). In addition, strain DZ 47 grown on nutrient broth or glycerol alone shows a constitutive expression of the KDG kinase. Therefore, this strain carries a mutation belonging to the *kdgR* constitutive class. The mapping of this mutation (Table 9) shows that the *kdgR*$^+$ or *kdgR*$^-$ (ts) markers of the donors do not segregate at all from the *kdgA*$^+$ character selected.

These results are consistent with a deletion of the cluster *kdgA*, *edd*, *zwf* extending into *kdgR*.

Since a deletion in the regulator gene leads to the constitutive expression of the nondeleted operons, *kdgT* and *kdgK*, the *kdgR*$^+$ product seems to be essential to maintain the *kdg* regulon in a repressed state.

DISCUSSION

In addition to the physiological properties of the *kdgK* mutants reported previously, the study of their growth on KDG or either hexuronate has contributed to the discovery of a secondary pathway in *E. coli*. The intermediates are still unknown. However, it seems reasonable that one or two steps would be enough to convert KDG to an intermediate in a known metabolic pathway of *E. coli*. For instance, a decarboxylation of carbon 1 would yield 2-deoxy-ribose, which could be metabolized in *E. coli* by an inducible pathway (13, 17). A second possibility is two successive oxidations of carbon 6, giving 2-keto-3-deoxy-glucarate, an intermediate of the aldarate pathway in *E. coli* (D. C. Fish, Ph.D. thesis, Univ. of Michigan, Ann Arbor).

As is well known, negative mutants provide a useful tool for investigation of new metabolic pathways; nevertheless, conclusions must be cautiously drawn. KDPG aldolase-negative mutants are a good example—these mutants do not grow on gluconate or hexuronate, although secondary pathways are available for both substrates—gluconate is metabolized by the pentose-phosphate route in the wild type (19, 33) and hexuronate is metabolized by the by-pass reported in this paper. The absence of growth on these compounds is related to the toxicity of

TABLE 8. *Transduction of the kdgR locus (kdgR [ts] mutations)*[a]

Donor	Recipient	Selected markers	No. analyzed	Inheritance of unselected markers (%)			
				kdgA$^-$	*edd*$^+$	*kdgR*$^-$ (ts)	
C 31 (*kdgR* [ts])	AD 3141 (*kdgA*, *edd*)	*kdgA*$^+$	516	—	96	54.5	
C 41 (*kdgR* [ts])	AD 3141	*kdgA*$^+$	152	—	98.5	67.5	
C 51 (*kdgR* [ts])	AD 3141	*kdgA*$^+$	152	—	96	58.5	
C 81 (*kdgR* [ts])	AD 3141	*kdgA*$^+$	152	—	97	59	
C 91 (*kdgR* [ts])	AD 3141	*kdgA*$^+$	152	—	95	49.5	
				edd$^+$	*kdgR*$^-$	*kdgR*$^-$ (ts)	*oldD*$^-$
CS 391 (*kdgR*, *oldD*)	CAD 91 (*kdgR* [ts]), *kdgA*, *edd*)	*kdgA*$^+$	220	98.5	59	41	22

[a] The different *kdgR* alleles were distinguished by their phenotype on KDG (1 mg/ml) minimal medium: *kdgR*$^-$ (ts) is Kdg$^+$ at 42 C and Kdg$^-$ at 28 C; *kdgR*$^-$ is Kdg$^+$ at both temperatures; *kdgR*$^+$ is Kdg$^-$ at both temperatures.

TABLE 9. *Transduction—mapping of the kdgR*$^-$ *mutation of the DZ 47 strain Δ (edd, zwf, kdgA)*

Donor	Recipient	Selected markers	No.	Inheritance of unselected markers (%)		
				zwf$^+$	*edd*$^+$	*kdgR*$^+$
K 63	DZ 47 (*kdgA*, *zwf*, *edd*, *kdgR*)	*kdgA*$^+$	150	100	100	100
				zwf$^+$	*edd*$^+$	*kdgR*$^-$ (ts)
C 91 (*kdgR* [ts])	DZ 47	*kdgA*$^+$	60	100	100	100

KDPG as has been indicated previously (6, 7, 19). When the formation of the toxic compound is prevented by an earlier block, like *edd*⁻ for the gluconate pathway (19, 33) or *kdgK* for the hexuronate pathway (this paper), the secondary pathway is in each case revealed.

The generation time of 3 to 4 h, when KDG or galacturonate is metabolized only by the secondary pathway, does not reflect exactly the importance of this bypass. The growth rate is obviously measured on a kinaseless strain which overproduces KDG, and this compound has a slight inhibitory effect on the growth—the growth rates of these mutants are, respectively, 75 and 135 min on glycerol and glycerol plus galacturonate. Only isotopic experiments with the wild type will give us the relative importance of the two pathways metabolizing hexuronates from KDG.

The location of the *kdgK* mutation completes the genetic study (20, 23) of the KDG degradative pathway. Although no altered KDG kinase has been detected among a number of strain KO1 revertants (spontaneous or induced by ultraviolet light), it is likely that the *kdgK* locus is the structural gene of this enzyme.

The properties and mapping of the *kdgR* constitutive mutants have contributed to understanding the genetic control of the KDG degradative pathway. We have recently shown that these pleiotropic mutations affect neither the control of the enzymes generating KDG from the hexuronates nor the control of the enzymes of the gluconate pathway, gluconate uptake, gluconate kinase, or gluconate-6-P dehydrase (22). Nor is the enzyme(s) degrading KDG by the secondary pathway under the control of the *kdgR* product. Indeed, the strain CAK 101 carrying the constitutive allele *kdgR* is able to metabolize KDG by the new pathway only when it has been previously induced (Fig. 3). Moreover, it has been shown (20) that [^{14}C]KDG taken up by glycerol-grown CAK 101 is totally chased out by an excess of [^{12}C]KDG. So far, we have only observed that the unit of regulation controlled by the *kdgR* product consists of KDG uptake, KDG kinase, and KDPG aldolase.

As far as the nature of the genetic control is concerned, the hypothesis of a negative control postulated recently (21) is strengthened by the present study. The evidence that *kdgR* is a repressor rests on: (i) the selection at high frequency (10^{-5} to 10^{-6}) of pleiotropic constitutive mutants on KDG which behaves like a noninducing substrate (7) (such a property has been reported in other systems negatively controlled [3, 28, 32]); (ii) the properties of the *kdgR* (ts) mutants; according to the negative control model (11), a thermosensitive mutation in the regulator gene leads to the constitutive phenotype at high temperature but not at low (3, 12, 9, 30) (such a phenotype is encountered with the *kdgR* [ts] mutation), whereas in a positive control system as specified by the arabinose operon in *E. coli*, a thermosensitive mutation in the *araC* gene leads to a negative expression of the operon at 42 C (10); (iii) the observation that *kdgR*$^+$ is trans dominant to the *kdgR* constitutive allele; (iv) the fact that a strain carrying a deletion of the *kdgR* gene has lost the control of the nondeleted operons *kdgT* and *kdgK*.

Although the three enzymes degrading KDG are all controlled by the *kdgR* product, we have shown that a strong decoordination of the induction pattern can occur (22). Indeed, when gluconate is the sole carbon source, only the aldolase is derepressed, whereas the kinase and aldolase are also induced on hexuronate-grown cells. Finally, growth on KDG leads to the derepression of the three necessary enzymes.

It is noteworthy that this derepression, depending on the carbon source, is perfectly adapted to provide an economy of protein synthesis. It seems reasonable to assume that these different levels in the degree of repression of the three operons by the *kdgR* product depend on difference in operator structures. The factors of derepression (activity of *kdgR* constitutive strain/activity of noninduced wild type) of these operons (22) are increasing in the order *kdgA*, *kdgK*, *kdgT*. One can assume that the inducer of aldolase formed by the metabolism of gluconate is too weak to derepress the *kdgK* and *kdgT* operons. However, KDG, a stronger inducer, is able to derepress *kdgA* and *kdgK* but has little effect on the most repressed operon—*kdgT*. Moreover, it is interesting to observe that the sensitivity of these operons to catabolic repression is increasing in the same order as the repression exerted by the *kdgR* product.

If the *kdgT* product has become an unnecessary activity (free KDG not encountered in nature), it is possible that this operon has evolved towards a repressed state (the catabolic repression and the high affinity of the *kdgR* product for this operon are two additional effects).

In this pathway noncoordinate control is of value to the cell; such a system of control may most easily evolve in a situation where the structural gene for each enzyme has its own independent operator and promotor. A regulon seems to us to be more suitable for the evolution

of an appropriate fine control of enzymes involved in several pathways than a polycistronic unit.

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