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Allantoin Transport in *Saccharomyces cerevisiae* Is Regulated by Two Induction Systems

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We show that the allantoin transport system of *Saccharomyces cerevisiae* responds to two induction systems, one mediated by allophanate or its analog oxalurate and the other mediated by allantoin or its analog hydantoin acetate. The effects of the two inducers were additive in strain M85. Like other allantoin pathway genes, oxalurate-mediated induction of allantoin transport required a functional *DAL81* gene product. Hydantoin acetate-mediated induction of the system, on the other hand, occurred normally in *dal81* mutants. This suggests that induction was not only mediated by two separate inducers, but also involved different regulatory proteins. Induction is probably a transcriptionally regulated process, because addition of hydantoin acetate or oxalurate to the culture medium increased the steady-state levels of mRNA encoded by a gene required for allantoin transport (*DAL4*).

Allantoin, an intermediate in the degradation of adenine and guanine, is a major nitrogen source for *Saccharomyces cerevisiae* growing in its natural habitat. This compound enters the cell by way of a low-K_m (14), inducible active transport system (16). Operation of the transport system requires the *DAL4* gene product. This is evidenced by the observations that *dal4* mutant strains are unable to use allantoin as a nitrogen source and have lost the ability to accumulate it in the cell (4, 14). Regulation of this system contrasts with that of the pathway enzymes, even though allantoin transport may be considered the first step of degradation. Several of the pathway enzymes have been shown to be induced by allophanate or its analog oxalurate (OXLU), but not allantoin (17). OXLU-mediated induction requires a functional *DAL81* gene product, as demonstrated by its loss in *dal81* mutants (19). Production of the allantoin transport system, on the other hand, has been found to be induced 10- to 15-fold by allantoin or its analogs hydantoin and hydantoin acetate (HAA) (16).

At the same time that we found that allantoin induced its own transport, we noticed that OXLU was able to support a modest (threefold) induction of the allantoin transport system (16). However, we were unable to ascertain whether OXLU was functioning as an inducer or was acting indirectly by mobilizing the large pools of allantoin normally sequestered in yeast cell vacuoles (21). Therefore, little was made of the observation.

The isolation of regulatory mutants, which have defects in the allophanate-mediated induction process, and the acquisition of an mRNA probe for the *DAL4* gene permitted us to reopen the issue of allantoin transport regulation. The results reported in this paper suggest that production of at least some components participating in allantoin transport is regulated by two separate control systems, each possessing its own inducer. One control system responds to the presence of allantoin, as previously documented, while the other responds to allophanate and requires a functional *DAL81* gene product just as the other allantoin degradative functions.

(Preliminary accounts of this work has already appeared [T. Cooper, V. Turoscy, H. J. Cho, and H. S. Yoo, Abstr. Twelfth Int. Conf. Yeast Genet. Mol. Biol. 1984, p. 166].)

MATERIALS AND METHODS

Strains and culture conditions. The strains of *S. cerevisiae* and *Escherichia coli* used in this work are listed in Table 1. Strains M1097, M1487, M1095, M970, and M1407 are all derivatives of strain E1278b (the wild-type strain used by Wiame et al.), while strain M85 was derived from the M25 background. All other strains contain hybrid genetic backgrounds other than M25 or Σ1278b. Even though they are genetic hybrids, strains M1298 and M1300 are similar to strains of the M25 genetic background in that they are resistant to nitrogen catabolite repression when grown in glucose-ammonia medium. All of the culture conditions for growth and transformation have been described in detail by Sumrada and Cooper (18). Proline (0.1%) was used as a nitrogen source in the culture used for transport assays. OXLU (0.25 mM) or 1 mM HAA was provided as inducer when indicated throughout this study.

Allantoin uptake assays. Allantoin transport assays were performed by the procedures described by Sumrada and Cooper (18). The only radioactive allantoin available for studies of allantoin transport possessed very low specific radioactivity (14, 16). During early experiments that defined and characterized allantoin transport, the low resolution of the assay was not a liability. However, the extended times required to accumulate measurable amounts of radioactivity (40 to 120 min compared with 0.5 to 2.0 min normally used for the assay of urea transport [15]) generated a problem that went unnoticed until the present work. Cells used for accumulation measurements were derived from growing or healthy resting cultures. When allantoin was added to the medium, it accumulated as reported previously (14, 16), but the 40- to 120-min assay times were sufficient for accumulated radioactive allantoin to function as an inducer of the system. Therefore all allantoin measurements are a sum of initial allantoin uptake plus uptake observed in response to induction by allantoin accumulated during the first several

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TABLE 1. Strains used in this work

Strain	Genotype[a]
S. cerevisiae	
VT78	*MATa trp1 dal4-78* (derived from mutagenesis of RH218)
M1-2B	*MATα ura3-52 trp1-258*
M25	*MATa his6 ura1 lys1* / *MATα ade6 leu1*
M85	*MATa his6 ura1 dal1-16* / *MATα ade6 leu1 dal1-16*
M970	*MATa lys5* / *MATα lys2*
M1095	*MATa lys5 dal1-224* / *MATα lys2 dal1-224*
M1097	*MATa lys5 dal1-224 dal80-1* / *MATα lys2 dal1-224 dal80-1*
M1298	*MATa trp1 dal4-78* / *MATα trp1 dal4-78*
M1300	*MATa trp1 dal4-79* / *MATα trp1 dal4-79*
M1372-8a	*MATa trp1 his6 ura3-52 dal4-33*
M1372-18b	*MATα trp1 his6 ura3-52 dal4-33*
M1407	*MATa lys2 dal81-2* / *MATα lys5 dal81-2*
M1488-8a	*MATa trp1 dal1-16 dal4-1175*
M1487	*MATa lys5 dal1-224 dal81-2* / *MATα lys2 dal1-224 dal81-2*
RH218	*MATa trp1 mal SUC2 CUP1 gal2*
E. coli:	
SF8	*hsdR hsdM recB recC lop-11 supE44 gal-96* Sm^r *leuB6 thi-1 thr*
HB101	*hsdR hsdM recA13 supE44 lacZ24 leuB6 proA2 thi-1* Sm^r

[a] *dal4-78*, *dal1-16*, *dal1-224*, *dal4-79*, *dal4-33*, and *dal4-1175* are equivalent to *dal4-VT78*, *dal1-N16*, *dal1-GC224*, *dal4-VT79*, *dal4-N33*, and *dal4-N1175*, respectively.

minutes of assay. In this regard, it is important that induction of allantoin pathway enzymes has been shown to reach steady-state rates of synthesis approximately 3 min after the inducer was added at 30°C (7).

Manipulation of strains and plasmids. All methods used in the isolation, analysis, and use of the plasmids described in this work were those described by Sumrada and Cooper (18).

Northern blot analysis. Poly(A)$^+$ RNA from wild-type strains (M25 or M970) or a *dal81* mutant (M1407) was isolated by the method of Carlson and Botstein (1). Cultures used for RNA isolations were grown in Wickerham (17) minimal medium with ammonia, proline, or asparagine (0.1%) as the sole nitrogen source. Northern blotting (RNA blotting) and hybridization methods were as described by Yoo et al. (20). Plasmid pVT6, rendered radioactive by nick translation, was used as a hybridization probe.

RESULTS

OXLU-mediated induction of allantoin transport in wild-type and mutant strains. The issue to be resolved in this work is whether allantoin transport is induced by OXLU. If OXLU-mediated increases in allantoin transport resulted from indirect mobilization of sequestered allantoin pools, then the same levels of OXLU-mediated induction should occur in wild-type and *dal81* mutant strains. If, on the other hand, OXLU induces allantoin transport in a manner similar to its induction of other allantoin system enzymes, then a functional *DAL81* product should be required for the effect to be observed.

Allantoin accumulation increased after a 20-min lag in wild-type cells (strain M1095) grown in the absence of inducer (Fig. 1A, solid circles). This lag was probably generated by the time required for induction to occur as a result of adding [^{14}C]allantoin to the assay medium as described in Materials and Methods. When wild-type cultures were grown in medium containing OXLU as an inducer (Fig. 1A, open circles), the lag disappeared, a result expected if full induction occurred prior to the beginning of the assay. When cells were grown in the presence of OXLU, the absence of a lag was completely dependent on a functional *DAL81* gene product. The lag remained even though OXLU

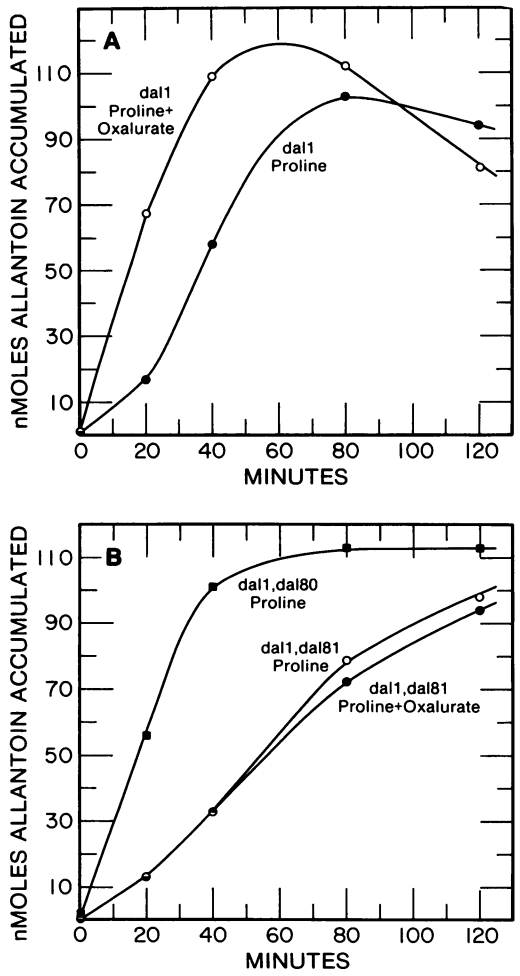

FIG. 1. Induction of allantoin transport by OXLU in wild-type and mutant strains of *S. cerevisiae*. Cultures of (A) strain M1095 (*dal1*) (○ and ●) and (B) strain M1487 (*dal1 dal81*) (○ and ●) or M1097 (*dal1 dal80*) (■) were grown overnight to a cell density of 40 Klett units in glucose-proline medium with or without 0.25 mM OXLU as an inducer. At that time, samples were harvested by filtration, suspended in prewarmed glucose-proline medium, and allowed to equilibrate for 5 min at 30°C. Radioactive allantoin (specific activity, 0.2 μCi/μmol) was added to each culture at a final concentration of 0.1 mM. Samples were removed thereafter at the times indicated and assayed for allantoin accumulation as described in Materials and Methods. All strains used for the assay of allantoin transport contained the *dal1* mutation to dissociate uptake of allantoin from its metabolism. This mutation results in the loss of allantoinase activity. Therefore, throughout this work, a *dal1* mutant was considered to be wild type. Data are expressed in nanomoles of allantoin accumulated per milliliter of culture.

was present in the culture medium of a *dal81* mutant (strain M1487), i.e., putative OXLU-mediated induction did not occur in the *dal81* mutant (Fig. 1B).

We repeated this experiment with a *dal80* mutant (strain M1097). It has been shown that the allantoin-degradative enzymes are produced at high, constitutive levels in *dal80* mutants even though inducer (allophanate or OXLU) is absent (3). A high rate of allantoin transport occurred in the *dal80* mutant, even though OXLU was not added to the culture medium; the lag was absent (Fig. 1B). In other words, putative OXLU-mediated induction of allantoin transport exhibited the same responses to mutation of the *DAL80* and *DAL81* genes as the allantoin-degradative enzyme genes (3, 19).

HAA mediated induction of allantoin transport in wild-type and mutant strains. Sumrada et al. demonstrated that allantoin transport was induced by allantoin or its analog HAA (16). The fact that OXLU was also capable of serving this function prompted the question of whether putative induction by the two compounds was additive. We answered this question by growing strain M85 (*dal1*) in glucose-proline medium and determining the effects of adding OXLU or HAA, or both, to the cultures. Addition of either OXLU or HAA to the culture medium of this wild-type strain resulted in increased rates of allantoin accumulation (Fig. 2). Addition of both compounds resulted in a rate of allantoin uptake that was roughly equal to the sum of the rates observed when the compounds were added separately.

To ascertain whether the *DAL81* gene product was required for allantoin- or HAA-mediated induction of allantoin transport, we repeated the experiment described in Fig. 1B with a *dal81* mutant (strain M1487). Growth of the *dal81* mutant in the presence of HAA eliminated the lag in allantoin accumulation (Fig. 3, solid squares and open circles). In contrast, OXLU was not able to eliminate the lag in this strain and hence served as a negative control for the experiment. Addition of HAA resulted in the same level of transport activity as a combination of HAA and OXLU. We concluded that a functional *DAL81* gene product was required for induction of allantoin transport by OXLU but not allantoin or its nonmetabolized analog, HAA.

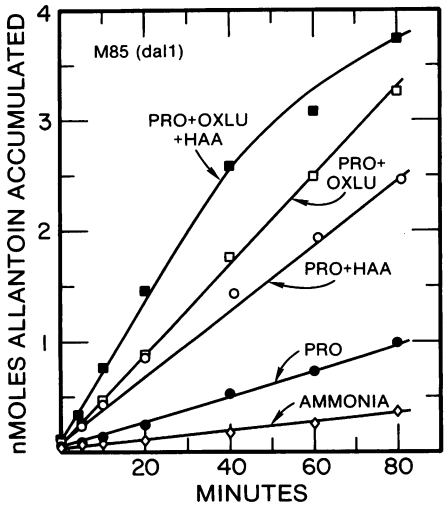

FIG. 2. Induction of allantoin transport by OXLU and HAA. Strain M85 (a *dal1* mutant in an M25 genetic background) was grown to a cell density of 45 Klett units in glucose-proline (●) or glucose-ammonia (◇). Compounds to be tested for induction (0.25 mM OXLU [□], 1 mM HAA [○], or the two compounds together [■]) were added to proline medium for the entire growth period. Allantoin transport was measured as described in Materials and Methods.

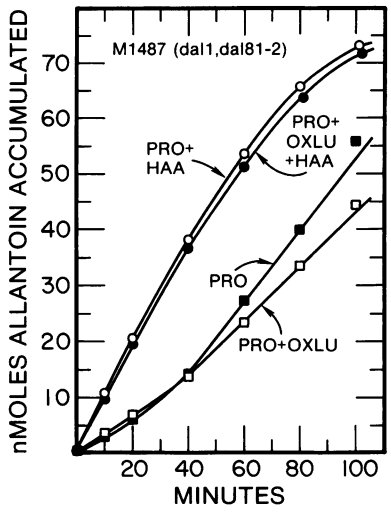

FIG. 3. Induction of allantoin transport in a *dal1 dal81* mutant strain by OXLU or HAA; or both. A culture of strain M1487 (*dal1 dal81-2*) was grown (to a cell density of 45 Klett units) in glucose-proline medium (PRO) in the presence of 0.25 mM OXLU (PRO+OXLU), 1 mM HAA (PRO+HAA), or both (PRO+OXLU+HAA). Samples were assayed for allantoin transport as described in the legend to Fig. 1.

Isolation of the *DAL4* gene. The above data suggested that induction of allantoin transport was controlled by two distinct regulatory systems. Allantoin or HAA served as inducer for one system, and allophanate or OXLU served this function for the other. The most rigorous means of testing this hypothesis was to isolate a gene encoding a component of the transport system and use it to assay mRNA production that occurred in response to addition of HAA or OXLU. Since *DAL4* is presently the only gene known to be required for allantoin transport, we isolated it by transformation-complementation methods (20). We used the genomic plasmid bank prepared by Nasmyth and Reed (11) to transform a *dal4* mutant (strain M1298) derived from strain RH218. Only a single transformant was observed. We isolated the plasmid contained in this transformant and found that it was able to transform a *dal4* mutant to the wild-type phenotype at high frequency. Its restriction map (plasmid pVT78) is shown in Fig. 4.

We had previously isolated plasmids pTC12 and pTC7, which were able to complement mutations in the *DAL1* and *DAL2* genes, respectively (20; R. Buckholtz and T. G. Cooper, manuscript in preparation). Since the *DAL4* gene is situated between *DAL1* and *DAL2*, we suspected that an area of restriction site homology might be found between segments of plasmids pTC7 and pTC12 and that one of the plasmids would complement *dal4* mutations. Only a single potentially common site (*Sac*I) was observed near the ends of both plasmids, but plasmid pTC12 (Fig. 5) was able to transform *dal4* mutants to a wild-type phenotype (growth on minimal-allantoin medium) at high frequency. With this result, we had identified two plasmids able to complement *dal4* mutations at high frequency. Their restriction maps were completely different (compare plasmids pVT78 and pTC12 in Fig. 4 and 5). This suggested that at least one of the

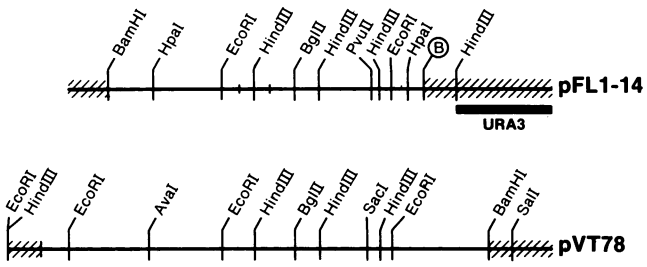

FIG. 4. Restriction endonuclease map of plasmids pFL1-14 and pVT78. The restriction site map of plasmid pVT78 was determined by methods described previously (18). The map of plasmid pFL1-14 is that published by Chevallier (2). *Hpa*I and *Pvu*II sites were not mapped for plasmid pVT78. Likewise, *Ava*I and *Sac*I sites were not determined for the published map of plasmid pFL1-14.

plasmids did not carry the *DAL4* gene, but a suppressor of the *dal4* mutation.

In view of this situation, our first objective was to identify which of the complementing plasmids contained DNA homologous to that of the *DAL4* gene on the right arm of chromosome IX. The entire insert from plasmid pVT78 and a 2.5-kilobase (kb) *Xho*I-*Sal*I fragment from the right end of plasmid pTC12 were subcloned into the integrative vector YIp5 yielding plasmids pVT1 and pRB2 (13). These constructions were used to transform strain M1372-8a to a Ura^+ phenotype. Stable transformants were crossed to strain M1372-18b, and the resulting diploids were sporulated. Segregation patterns of the markers included in these crosses are shown in Table 2. The *URA3* marker carried on plasmid

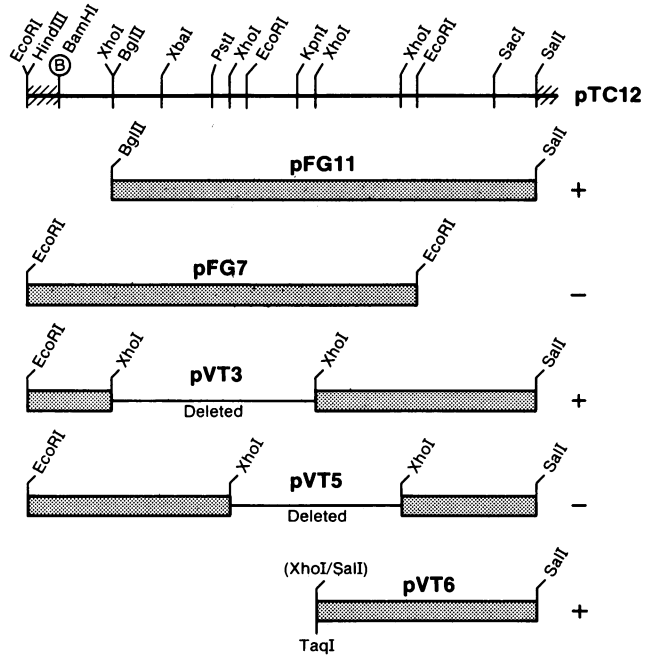

FIG. 5. Localization of the *DAL4* gene on plasmid pTC12. The designated portions of plasmid pTC12, whose restriction site map appears across the top of the figure, were recloned into the autonomously replicating vector YRp7. Each of the constructions was then used to transform a *dal4* mutant strain (M1298 or M1300) and tested for its ability to complement the mutation it contained. Symbols: +, high-frequency transformation with allantoin as the sole nitrogen source; −, lack of transformation.

pVT1 (derived from plasmid pVT78) showed no linkage with the *lys1* or *his6* markers of chromosome IX (crosses 1 and 2). In contrast, the *URA3* marker of plasmid pRB2 cosegregated with both *lys1* and *his6*, indicating that DNA carried on plasmid pTC12 had integrated into the right arm of chromosome IX. The same result was observed whether circular or linear DNA was used in the transformation. The genetic distance calculated between the *URA3* and *lys1* loci in the integrant was 2.6 centimorgans (cM) compared with 6.5 cM reported between the *dal4* and *lys1* loci (4). This result supports the suggestion that DNA contained in plasmid pTC12 derived from the genomic *DAL4* locus.

Localization of the *DAL4* gene. We located the minimum sequence required for complementation of *dal4* mutations by subcloning various fragments of plasmid pTC12 and testing their complementing ability. The smallest fragment capable of complementation was a 2.5-kb *Xho*I-*Sal*I fragment from the right-hand side of plasmid pTC12 (Fig. 5). Removal of sequences to the right of the right-hand-most *Xho*I and *Eco*RI sites on plasmid pTC12 resulted in loss of complementing ability. The fact that plasmid pVT6 complemented *dal4* mutations suggested that the entire *DAL4* gene was carried on this plasmid.

The ability of plasmid pVT6 to complement the biochemical function lost in *dal4* mutants (allantoin accumulation) was tested by assaying transformants of strain M1488-8a. No transport activity was observed in the recipient strain transformed with the vector plasmid (YRp7), regardless of whether it was grown in the presence of inducer (allantoin) (Fig. 6A). When plasmid pVT6 was used instead, inducible allantoin transport activity was observed (Fig. 6A). Allantoin transport activity was also found in strains transformed with plasmid pVT78 (Fig. 6B). However, in contrast to results with plasmid pVT6, allantoin transport activity supported by plasmid pVT78 was constitutively produced.

TABLE 2. Linkage of the integrated *URA3* gene to *his6* and *lys6* on chromosome IX[a]

Integrated plasmid	No. of asci analyzed	Gene pair	No. of asci		
			Parental ditype	Nonparental ditype	Tetratype
pVT1[b]	41	*URA3-lys1*	7	9	25
		URA3-his6	14	14	13
		his6-lys1	9	2	30
PVT1[c]	35	*URA3-lys1*	11	10	14
		URA3-his6	12	11	12
		his6-lys1	13	2	20
pRB2[c]	38	*URA3-lys1*	36	0	2
		URA3-his6	16	2	20
		his6-lys1	14	2	22
pRB2[c]	39	*URA3-lys1*	37	0	2
		URA3-his6	10	1	28
		his6-lys1	8	1	30

[a] Strain M1372-8a was transformed with the plasmids shown, and stable integrants were selected as described in the text. Individual integrated colonies were mated to strain M1372-18b, and diploid strains were then selected and sporulated by standard methods. Plasmid pVT1 contains the entire insert of plasmid pVT78 (*Cla*I-*Sal*I) into integrative vector YIp5 (18). Plasmid pRB2 contains the 2.5-kb *Xho*I-*Sal*I fragment from the right end of plasmid pTC12. Genetic distances were calculated with the formula of Perkins (12).

[b] Plasmid was integrated as a circular molecule.

[c] Plasmid was integrated as a linear molecule (plasmid was digested with *Sac*I).

The fact that plasmid pVT78 supported constitutive production of allantoin transport activity suggested that suppression of the *dal4* mutation by this plasmid did not derive from an informational suppressor. An informational suppressor would have suppressed the mutation but probably would not have altered normal regulation of the gene carrying the suppressible mutation. This suggested that plasmid pVT78 carried a gene that, when present in high copy number, was capable of physiologically suppressing the effects of *dal4* mutations. We recognized that restriction maps of plasmids pVT78 and pFL1-14 were homologous (Fig. 4). The latter plasmid has been reported by Chevallier to carry the *URAP* gene which encodes uracil permease (2).

Regulation of *DAL4* gene expression in wild-type and mutant strains. The cloned *DAL4* gene provided an opportunity to directly test whether its expression was induced by HAA, as previously suggested (16), and by OXLU, as suggested by the data in Fig. 1. These questions were addressed by growing a wild-type (strain M25) culture of *S. cerevisiae* in minimal glucose proline medium without added inducer or in the presence of either OXLU or HAA. Low levels of a 2.1-kb transcript were observed in the absence of inducer (Fig. 7, lane C). The level of this transcript increased markedly upon addition of the allantoin analog HAA to the culture medium (compare lanes C and D). Expression of *DAL4* was subject to nitrogen catabolite repression, as indicated by the diminished expression observed when ammonia replaced proline as the nitrogen source (compare lanes C and D with A and B). OXLU was also found to increase the level of the 2.1-kb transcript (compare lanes C and E). In fact, its addition to the medium elicited production of significantly higher *DAL4*-specific mRNA levels than did addition of HAA (compare lanes D and E). OXLU-mediated induction required a functional *DAL81* gene product. Very little *DAL4*-specific RNA was observed when a

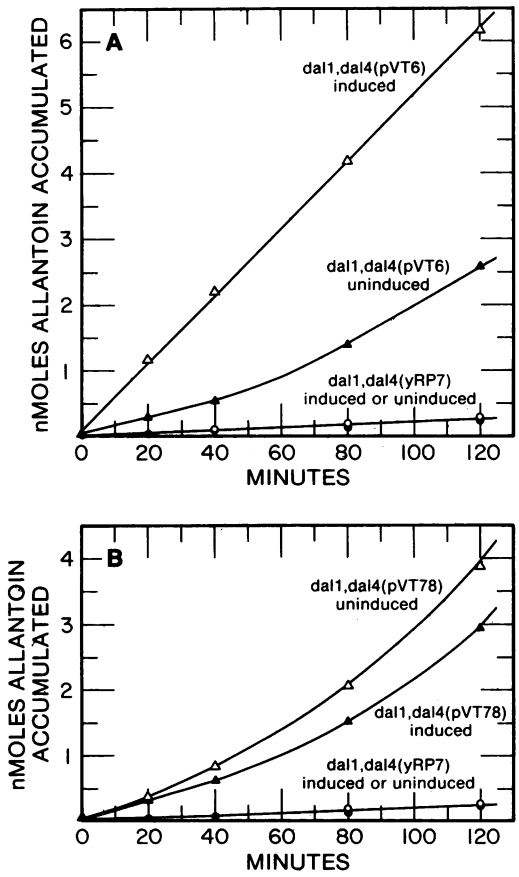

FIG. 6. Allantoin transport observed in *dal4* mutant strains transformed with various plasmids and grown in the presence or absence of inducer. The *dal1 dal4* strain (M1488-8a) transformed with plasmid pVT6 (Fig. 4) or pVT78 (Fig. 3) was inoculated in glucose-ammonia medium (uninduced) or glucose-ammonia medium containing 1 mM allantoin (induced) and grown overnight. At a cell density of 40 Klett units, a sample of each culture was harvested by filtration and transferred to an equivalent volume of glucose-ammonia medium. After equilibration for 5 min at 30°C, [^{14}C]allantoin was added to a final concentration of 0.1 mM. Samples were assayed as described in Materials and Methods. Data are expressed as nanomoles of allantoin accumulated per milliliter of culture.

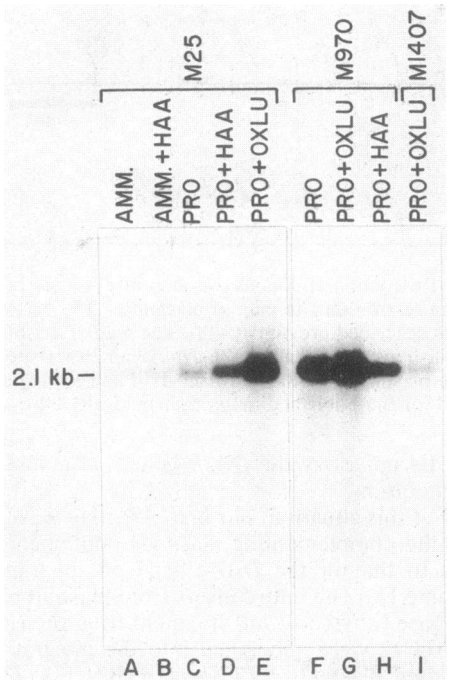

FIG. 7. Steady-state levels of *DAL4*-specific poly(A)$^+$ RNA observed in wild-type (strain M25, lanes A to E; strain M970, lanes F to H) or *dal81* mutant (strain M1407, lane I) cells grown in the presence or absence of HAA or OXLU. Strain M25 was grown overnight in glucose-proline medium to a cell density of 40 Klett units in the presence or absence of compounds being tested for their inducing ability. For strain M970, inducer was added when cultures reached 20 Klett units. One generation later, the cultures were harvested and processed as described in Materials and Methods. All poly(A)$^+$ RNA was isolated as described in Materials and Methods. Equal amounts (10 μg) of poly(A)$^+$ RNA, derived from each of the culture conditions described above and isolated as described in Materials and Methods, were resolved on formaldehyde-agarose gels and transferred to nitrocellulose paper. The *DAL4*-specific probe (plasmid pVT6) was made radioactive by nick translation. Hybridization conditions were as described previously (20). Plasmid pBR322 DNA digested with *Hin*fI and *Ava*I (2 to 3 pg) was included as size standards in lanes flanking the RNA samples. Two autoradiograms, derived from two different experiments, are shown in this figure. One autoradiogram contains lanes A to E, while the other contains F to I. This precludes quantitative comparison of RNA levels observed on one autoradiogram with those on the other. Lane designations: AMM or PRO, ammonia or proline was used as nitrogen source; +HHA or +OXLU, HAA or OXLU, respectively, was added as inducer.

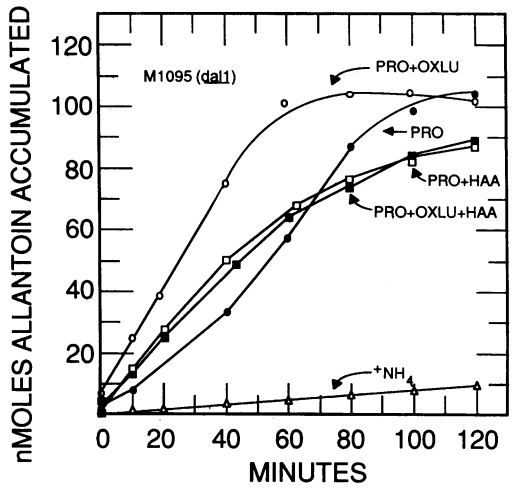

FIG. 8. Induction of allantoin transport in an Σ1278b genetic background. Cultures of strain M1095 (*dal1* mutant derivative of Σ1278b) were grown to a cell density of 45 Klett units in glucose-proline (●) or ammonia (△) medium. The test compounds, 0.25 mM OXLU (○), 1 mM HAA (□), or 0.25 mM OXLU + 1 mM HAA (■), were included in the proline medium as indicated. Samples of each culture were removed and assayed for allantoin uptake as described in the legend to Fig. 1.

dal81 mutant was grown in the presence of OXLU (compare lanes G and I).

Strain specificity of *DAL4* gene regulation. Figure 7, lanes C to H depict an unexpected, and potentially useful, characteristic of *DAL4* gene regulation: its strain specificity. RNA loaded into lanes A to E was isolated from cultures of wild-type strain M25, a relative of strain S288c. *DAL4* expression was highly inducible in this genetic background (16). In contrast, *DAL4* RNA was much less inducible in strain M970 (an isogenic diploid of Σ1278b, the wild-type strain used by Wiame et al.). The observed low-level induction did not derive from small differences in the amounts of RNA present on the blot. This possibility was eliminated by monitoring loading equivalence and transfer efficiencies with a probe (pVT78) containing three genes that did not respond to allantoin system control or regulatory mutations (data not shown). The observation of high inducibility for allantoin system gene expression in strain M25 and less inducibility in strain M970 has also been reported for the *DAL1* gene (8, 19).

Another difference between strain Σ1278b and M25 is their response to HAA. This allantoin analog is a reasonably strong inducer for strain M25 and its derivatives. In strain M970 and its derivatives, however, HAA is inhibitory to *DAL4* gene expression (compare Fig. 7, lanes F and H). Addition of HAA to strain M1095 (a derivative of Σ1278b) produced the same initial result as its addition to strain M85 (Fig. 2), i.e., the lag in allantoin accumulation was eliminated, indicative of induction (Fig. 8). Thereafter the culture containing HAA accumulated allantoin at a lower rate than observed with proline alone or in proline plus OXLU medium. Therefore the response observed in a Northern blot will be greatly influenced by the time for which HAA was present in the medium. RNA samples used in Fig. 7, lanes G and H, were derived from cells incubated with inducer for one generation. The source of inhibition is not easily identified and may be nonspecific, since addition of this compound to a culture slows its growth. The slowing of growth was almost absent in derivatives of strain M25, and inhibition was not observed at such early times (compare Fig. 2 and 8).

A final difference between the two strains can be seen by inspecting the quantitative levels of transport they support. The maximum values observed for 40 min of accumulation in strain M85, a derivative of strain M25, were ca. 4 to 5 nmol of allantoin accumulated per ml of culture (see data in Fig. 2 and reference 14). Similar measurements with strain M1407 and other derivatives of strain Σ1278b range up to 120 nmol of allantoin accumulated per ml of culture. This represents an approximately 25-fold increase. The biochemical reason for this difference is not known.

DISCUSSION

The data in this report suggest that *DAL4* gene expression is regulated by two separate control systems: the *dal81*-dependent, OXLU- or allophanate-mediated induction system and a second induction system for which allantoin and its analog HAA serve as inducers. The systems appear independent to the extent that the *DAL81* gene product, which is required for allophanate-mediated induction, does not seem to be needed for allantoin-dependent induction of *DAL4*.

The existence of two induction systems for allantoin transport makes good physiological sense. If allantoin transport were induced solely by allophanate, the cell probably could not induce production of the transport system. The metabolic buffering effect brought about by the need for five consecutive enzymatic reactions to synthesize the inducer (allophanate) would probably be sufficient to prevent the system from ever being turned on, regardless of how much environmental allantoin was available. This situation appears to have been solved by the evolution of a second induction system in which allantoin itself serves as inducer. If allantoin is encountered in the environment, transport by basal levels of activity would result in a small buildup of allantoin within the cell. This buildup would result in induction of the allantoin transport system, which would in turn result in accumulation of much larger quantities of the nitrogen source. At these higher levels, metabolism would occur by action of the rather substantial basal quantities of the allantoin-degrading enzymes. In the presence of a stable supply of external allantoin, sufficient allophanate would be synthesized to overcome the buffering effect generated by having the last intermediate of the pathway serve as inducer (5). Such a buildup of allophanate would trigger induction of the allantoin system to its full potential, including a substantial increase in allantoin transport activity needed to fully exploit available environmental allantoin.

The response of allantoin transport to two induction systems poses several interesting questions about the structure of *DAL4* upstream activation sequences and the potential interactions of regulatory proteins with them. The involvement of more than one regulatory protein in control of *DAL4* gene expression probably derives from structural requirements of the two inducers. Although the structures of allophanate, OXLU, allantoin, and HAA seem to be similar (Fig. 9), the resemblance may be more apparent than real. Crystallographic studies of allantoin suggest that the ureido group projects perpendicular to the plane of the hydantoin ring in much the same way that the tail of a scorpion extends above its body (10). OXLU and its close relative, biuret, on the other hand, are relatively planar (6).

Vexing problems initially generated by the fact that strains Σ1278b and M25 differ significantly in some aspects of

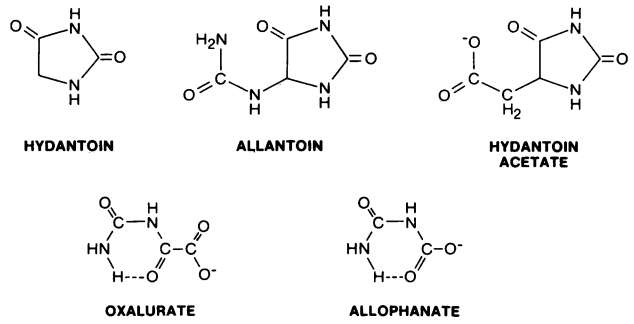

FIG. 9. Chemical structure of compounds able to induce production of the allantoin transport system (hydantoin, allantoin, or HAA) or the allantoin-degradative enzymes (OXLU or allophanate).

allantoin transport regulation may provide further insight into the subtleties of these regulatory systems. Three important differences are as follows. First, derivatives of strain M25 appear to be significantly more inducible than those of Σ1278b. This unrecognized difference between the two strains caused considerable controversy in early studies addressing control of allantoinase (*DAL1*) production (9). It is interesting that the characteristic appears again here. The pleiotropic lack of *DAL1* and *DAL4* inducibility suggests that it probably derives from alterations in the regulatory proteins that mediate this process rather than alterations in the target sequences of those proteins in the 5′ flanking regions of the *DAL1* and *DAL4* structural genes. Second, derivatives of Σ1278b are less responsive to induction by HAA and become inhibited by this compound much earlier than derivatives of strain M25. Third, derivatives of Σ1278b possess about 25-fold-higher uptake capacity than do derivatives of M25. The sources of these differences are not known. It is not even clear whether the observed increase in transport activity derives from alteration in the amount of *DAL4* gene product. It could just as easily result from overproduction or modification of another unknown but limiting protein component of the transport system. This lack of information highlights an important problem generated when uptake rates for a transport system are compared with the amount of a particular gene product or its cognate RNA. Transport is a highly complex process, involving participation of multiple proteins. Accumulation or initial transport rate assays measure the combined operation of all necessary components. The results of these assays are controlled by the limiting component of the entire transport system, be it a protein, the concentration of the transported ligand, or a membrane potential. Measurements of *DAL4* mRNA focus on only a single component without regard to whether it is the limiting one in the process. Therefore it is not surprising that accumulation levels and transport rates are not quantitatively reflected in component RNA levels. Comparison of the data in Fig. 2 and 7 reveal that the trends of the data are similar in the two assays but that the data lack absolute congruence.

Finally, the above studies depended, in part, on isolation of the *DAL4* gene. During the gene isolation phase of the work, we unexpectedly found a second gene that was able to suppress *dal4* mutations. The basis for this suppression remains unclear at present. However, we do know that suppression occurs only when the suppressor is present in more than two copies. Otherwise, *dal4/dal4* diploid strains would grow on allantoin. Suppression of *dal4* mutations appears to derive from the acquisition of an overproduced biochemical activity, rather than repair of the defective *dal4* gene product. An explanation for suppression may derive from the fact that the uracil permease gene is contained on the suppressing plasmid. If this gene is the suppressor, suppression may occur because the uracil permease possesses sufficient range in its substrate specificity to support low levels of allantoin uptake. When the *URAP* gene is present in high copy number (the gene is carried on a YRp7 vector which usually yields approximately a 7- to 10-fold increase in activity), there may be sufficient transport activity for physiological suppression to occur. However, one must view this explanation with some skepticism, because the insert of plasmid pVT78 hybridizes to three transcripts (R. Rai and H. S. Yoo, unpublished data). We have not directly determined whether it is the *URAP* gene which accounts for suppression of the *dal4* mutations.

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