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Mutants of *Escherichia coli* K-12 "Cryptic," or Deficient in 5'-Nucleotidase (Uridine Diphosphate-Sugar Hydrolase) and 3'-Nucleotidase (Cyclic Phosphodiesterase) Activity

I. R. BEACHAM, R. KAHANA, L. LEVY, AND E. YAGIL

Department of Botany & Microbiology, University College of Wales, Aberystwyth, SY23 3DA, United Kingdom; and Department of Biochemistry, The George S. Wise Center for Life Sciences, Tel Aviv University, Tel Aviv, Israel

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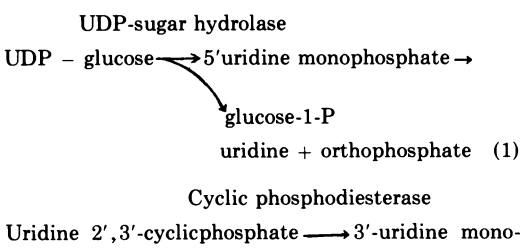
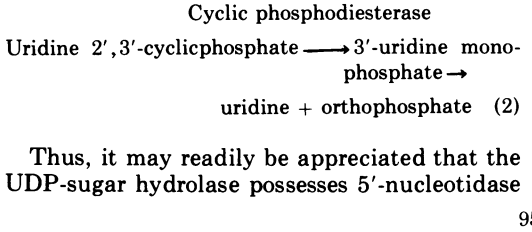
Mutants of *Escherichia coli* have been selected for the absence of 5'-nucleotidase (uridine diphosphate-sugar hydrolase) and 3'-nucleotidase (2',3'-cyclic phophodiesterase). Mutants selected for the absence of 5'-nucleotidase are of two kinds: those that lack detectable activity for the enzyme (Ush$^-$), and those that possess activity when cell extracts are assayed, but not when intact cells are assayed (cryptic; Crp$^-$). The latter class is probably identical to a type of mutant previously reported by Ward and Glaser. When mutants are selected for the absence of 3'-nucleotidase, Crp$^-$ mutants are also obtained. Thus far, however, mutants totally lacking this enzyme have not been found. The location on the genetic map of one *ush* mutation is at position 11 min and that of one *crp* mutation at approximately 67 min. In the *crp* mutant, 5'-nucleotidase and 3'-nucleotidase remain located in the periplasm. This mutant is also cryptic for alkaline phosphatase but not for acid hexose phosphatase. Treatment of cells with ethylenediamine-tetraacetate substantially alleviated crypticity. These data are discussed in terms of the organization of periplasmic enzymes and of the outer membrane as a permeability barrier.

Wild-type strains of *Escherichia coli* possess a 5'-nucleotidase and a 3'-nucleotidase (2, 10, 21). These enzymes are more correctly called uridine diphosphate (UDP)-sugar hydrolase and cyclic phosphodiesterase, respectively, since they catalyze the following reactions (2, 3, 10, 21, 22):





Thus, it may readily be appreciated that the UDP-sugar hydrolase possesses 5'-nucleotidase activity and the cyclic phosphodiesterase possesses 3'-nucleotidase activity. Since, in this paper, we more often utilize the nucleotidase activity of these enzymes, we will refer to them as such.

These enzymes are located in the periplasmic space (5, 10, 12, 13, 23, but compare 24). The question of whether certain periplasmic enzymes are bound to the surface of the cell or are free in the periplasmic space is an open one. Like most periplasmic enzymes, 5'-nucleotidase and 3'-nucleotidase activities are expressed by intact cells when the substrate is added externally (10, 17, 22, 33). In 1968, Ward and Glaser (33, 34) reported the isolation and characterization of mutants which are "cryptic" for UDP-glucose hydrolase activity. In the mutant cells, UDP-glucose hydrolase and 5'-nucleotidase activities are expressed in cell extracts but not in intact cell preparations. That is, in whole cells the enzyme activity is cryptic. The favored

interpretation of the properties of these mutants was that the enzyme is normally bound to the cytoplasmic membrane (or some other component of the cell envelope), but in the cryptic mutants the enzyme is not bound correctly, with resulting loss of activity in whole cells (33, 34). An alternative hypothesis (33) is that there is a permeability defect in the outer membrane, such that the substrate is inaccessible to the enzyme.

The work in this report was directed towards a better understanding of the cryptic mutants. We describe a direct selection for mutants either cryptic for, or lacking in, 5'-nucleotidase activity, genetic mapping of the mutants, and their properties.

MATERIALS AND METHODS

Bacterial strains. Table 1 lists the bacterial strains used; all were *E. coli* K-12 strains.

Media and growth conditions. The cells were grown at 37 C in appropriately supplemented M9 medium (28) with glucose (0.2%) as carbon source. For acid hexose phosphatase determination, Casamino Acids (1%; Difco) were used as carbon source (8). For the induction of β-galactosidase, the cells were grown for one generation in the presence of 1 mM isopropyl-β-D- thiogalactoside (IPTG). For the derepression of alkaline phosphatase, cells were grown in supplemented T medium (9) with 1 mM KH_2PO_4 and were, thereafter, transferred for overnight growth to the same medium without inorganic phosphate.

Preparation of cells for enzymatic determinations. Either logarithmically growing or stationary-phase cells were washed three times with equal volumes of 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.0) containing 0.03 M NaCl, and resuspended in 0.1 M Tris-hydrochloride buffer (pH 8.0) to give a cell density of approximately 6×10^9 cells per ml. A sample of the cell suspension was disintegrated by sonic oscillation and centrifuged for 20 min at 12,000 × *g*. The supernatant fluid (cell extract) was used for enzyme assays.

Bacterial conjugation. Bacterial conjugation was performed as described previously (19). The conjugation time varied according to each experiment. Counter selection of the Hfr parent was either with streptomycin (0.1 mg/ml) or with bacteriophage T6 (multiplicity of 50 to 100).

Spheroplast formation and osmotic shock treatment. Formation of spheroplasts and osmotic shock treatment were as previously reported (4).

Enzyme assays. 5'-Nucleotidase (EC 3.1.3.5) was assayed by the method of Nossal and Heppel (25), by measuring the release of inorganic phosphate (P_i) from adenosine 5'-monophosphate (5'-AMP). The reaction mixture (0.9 ml) contained 83 mM acetate buffer (pH 6.0), 5.0 mM $CoCl_2$, 16.0 mM $CaSO_4$, 0.33 mM 5'-AMP, and 0.05 ml of the enzyme source (equivalent to approximately 3.10^8 cells). When cell extract was assayed, the reaction mixtures were heated prior to the addition of 5'-AMP, for 10 min at 45 C to inactivate the intracellular inhibitor of 5'-nucleotidase (23). After 6 min of incubation of the whole reaction mixture at 37 C, the reaction was stopped with 0.1 ml of 0.5 N HCl in an ice bath. One enzyme unit (EU) is defined as nanomoles of P_i released per minute.

UDP-glucose hydrolase. Uridine diphosphate sugar hydrolase was assayed by measuring the disappearance of UDP-glucose using UDP-glucose dehydrogenase, under the conditions described by Ward and Glaser (34). The reaction mixture was heated prior to assay, as described for 5'-nucleotidase.

3'-Nucleotidase. 3'-Nucleotidase (EC 3.1.3.6) was assayed by the method of Neu and Heppel (21; see also 2), by measuring the release of P_i from 3'-AMP. The reaction mixture (0.9 ml) contained 83 mM sodium acetate buffer (pH 6.0), 5.0 mM $MgSO_4$, 1 mM $CoCl_2$, 0.33 mM 3'-AMP, and 0.1 ml of the enzyme source (equivalent to approximately 6.10^8 cells). The reaction was stopped after 40 min with 0.1

TABLE 1. *Characteristics of E. coli strains*[a]

Strain	Sex	Genotype or phenotype	Source
AB1157	F^-	*thr ara leu proA lac tsx gal his strA xyl mtl arg thi*	E. A. Adelberg
AB1157-1	F^-	*thr ara leu proA lac tsx gal his strA xyl mtl arg thi upp*	*FU-resistant mutant* of strain AB1157
AB259-1	HfrH (*O thr lac gal*)	FU^r	Fu-resistant mutant of strain AB259
AT2455-1	HfrH (*O thr lac gal*)	*malA cysG thi* FU^r	FU-resistant mutant of strain AT2455
P4X-1	Hfr (*O proA thr xyl*)	FU^r	FU-resistant mutant of strain P4X

[a] Symbols are as recommended by Taylor and Trotter (32). *upp*, UMP pyrophosphorylase (uracil phorphoribosyl transferase). The order of transfer of genetic markers of the Hfr strains is given in parentheses, O designates the origin of transfer.

ml of 0.5 N HCl. EU are defined as with 5'-nucleotidase.

Cyclic phosphodiesterase. Cyclic phosphodiesterase was assayed in the same way as 3'-nucleotidase, except that adenosine 2',3'-cyclic phosphate (1 mM) was used as substrate.

β-galactosidase. β-Galactosidase was assayed by the method of Pardee et al. (26) using *O*-nitrophenyl-β-D-galactoside as substrate. One EU is defined as the amount of enzyme producing one absorbancy unit at 410 nm per minute.

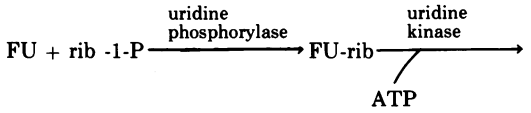
Alkaline phosphatase. Alkaline phosphatase was assayed as described by Schlesinger (30), using *p*-nitrophenyl phosphate as substrate. One EU is defined as with β-galactosidase.

Acid hexose phosphatase. Acid hexose phosphatase was assayed as described by Dvorak et al. (8), using glucose-6-phosphate as substrate. One EU is defined as with nucleotidase.

Determination of inorganic phosphate. Determination of P_i was performed as described by Ames and Dubin (1).

RESULTS

Mutant isolation. Mutants affected in nucleotidase activity were obtained directly by selecting for resistance to 5'-AMP or 3'-AMP, and 5-fluorouracil (FU). The method is a development of that used to select uridine phosphorylase (*udp*) and purine phosphorylase (*pup*) mutants (27). A mutant (AB1157-1) was first obtained which was resistant to FU (1 μg/ml). This mutant lacks uracil phosphoribosyl transferase (*upp*) activity (as measured by the method of Molloy and Finch [20]) and is thus blocked in the main route for incorporation of exogenous uracil or FU (27). If, however, a source of ribose-1-phosphate (rib-1-P) is made available, such mutants become resensitized to the same concentration of FU, since fluorouridylic acid (FUMP) may now be synthesized by the following route:



If a purine riboside (e.g., adenosine) is used as a source of rib-1-P, mutants are selected which cannot make rib-1-P from the riboside (and lack purine phophorylase), or cannot synthesize FUMP from FU and the rib-1-P (27). We have used 5'-AMP or 3'-AMP as a source of rib-1-P. Strains resistant to AMP and FU were selected from strain AB1157-1, and only those that remained sensitive to adenosine plus FU were retained. In addition, nucleotidase mutants were found to be unable to grow with the respective nucleotide as a sole carbon source. Table 2 summarizes the properties of these mutants.

Table 3 shows the 5'-nucleotidase and 3'-nucleotidase activity in the mutant strains, assayed by using cell-free extracts and whole cells. It may be seen that two kinds of mutant were obtained: (i) mutants lacking any detectable 5'-nucleotidase activity (represented by 5A-1) and which possesses normal 3'-nucleotidase activity (we assume these to be mutants in the structural gene for 5'-nucleotidase and designate them *ush* [UDP-sugar hydrolase]); (ii) mutants represented by 3-4 and 5-70, in which both 3'- and 5'-nucleotidase activities are greatly reduced in intact cells only, while cell extracts show normal activity. Thus, these mutants are "cryptic" (see reference 33) and, until the defect is more defined, we designate them *crp*. It should be emphasized that these mutants are cryptic for both 3'- and 5'-nucleotidase activity when the selection is performed with either 3'- or 5'-AMP (Table 3).

We have not yet been able to isolate a mutant totally defective in 3'-nucleotidase. An experiment in which we selected for resistance to FU plus 2'3'-cyclic-AMP was also unsuccessful in this regard; only Crp⁻ mutants were obtained.

It may be noted here that the 5'-nucleotidase mutants also lack UDP-glucose hydrolase activity, and that the cryptic mutants are also cryptic for this activity and for 2'3'-cyclic phosphodiesterase activity (Table 4).

Genetic mapping. We are particularly interested in the lesion that causes crypticity for 5'- and 3'-nucleotidase. An important question is

TABLE 2. *Growth characteristics of mutants on various media*

Phenotype	Growth[a] on:				
	Minimal[b]	Minimal + FU	Minimal + FU + Ado	Minimal + FU + AMP	Minimal with AMP as carbon source
Wild type	+	−	−	−	+
Upp⁻	+	+	−	−	+
Pup⁻	+	+	+	+	−
Ush⁻ or Crp⁻	+	+	−	+[c]	−

[a] + and − indicate, respectively, growth ability or inability. Concentration of FU is 2μg/ml and for nucleotides is 1 mM. Abbreviations: Ado, adenosine; Crp, cryptic; FU, 5-fluorouracil; Ush, uridinediphosphate-sugar hydrolase.

[b] Containing appropriate supplements, glucose as the carbon source, except in the case of the last column.

[c] Resistance in the case of a Ush⁻ strain is only to 5'-AMP and not 3'-AMP.

TABLE 3. *Nucleotidase activities in mutants of E. coli*[a]

Strain	Selection medium	5'-Nucleotidase activity		3'-Nucleotidase activity		Phenotype
		Whole cells	Cell extracts	Whole cells	Cell extract	
AB1157-1	FU	30.9	37.8(18.5)	1.9	3.28	Wild type
5A-1	FU + 5'AMP	0	0	1.69	2.68	5'-Nucleotidase negative (Ush)
3-4	FU + 3'AMP	0	25.6	0.04	4.16	3'- and 5'-Nucleotidase cryptic (Crp⁻)
5-70	FU + 5'AMP	0.2	16.6	0.11	3.81	3'- and 5'-nucleotidase cryptic (Crp⁻)

[a] Values are expressed as enzyme units (see Materials and Methods). Figure is parentheses shows 5'-nucleotidase activity without prior heating.

TABLE 4. *UDP-sugar hydrolase and cyclic phosphodiesterase activity*[a] *in mutants of E. coli*

Strain	UDP-glucose hydrolase activity		2',3'-Cyclic phosphodiesterase activity	
	Whole cells	Cell extract	Whole cell	Cell extract
AB1157-1	8.9	2.5[b]	0.91	1.68
3-4	1.1	2.5	0.07	1.58
5A-1	0	0		

[a] Values are enzyme units (see Materials and Methods).

[b] We assume the low activity relative to whole cells in this experiment to be due to incomplete destruction of the intracellular inhibitor, though under the conditions used we obtain complete expression of 5'-nucleotidase.

whether the mutation is in the gene specifying the enzyme(s) affected, or if it is in a distinct locus.

In the following experiments, the inability of the Ush⁻ mutants to utilize 5'-AMP as a carbon source was used for selection of Ush⁺ recombinants. Preliminary mating experiments (data not shown) with strain 5A-1 indicated linkage to *lac* and *gal*. Figure 1 shows a "time of entry" experiment (14) in the cross Hfr AB259-1 × F⁻ 5A-1. The *ush* locus is clearly located between *lac* (10 min) and *gal* (17 min), and is about 1 min from *lac*, on the *E. coli* genetic map (32). Table 5 shows the segregation of markers among Ush⁺ Str^r recombinants; the closest linkage (95.4%) is between *ush* and *tsx* (11 mins).

Crosses with the Crp⁻ strain (3-4) using Hfr P4X-1 (Table 6) show linkage of *crp* to *xyl* (70 min) and *str-A* (64 min). Similar results are obtained if Crp⁺ recombinants are selected with 5'-AMP or 3'-AMP as carbon source.

These results suggest that the lesion in the cryptic mutant is a single one and differs from the *ush* mutation. Three revertants, able to grow with 5'-AMP as carbon source, were isolated from strain 3-4; enzyme assays showed that intact cells regained both 5'- and 3'-nucleotidase activity. We take this as further evidence that crypticity is caused by a single genetic lesion.

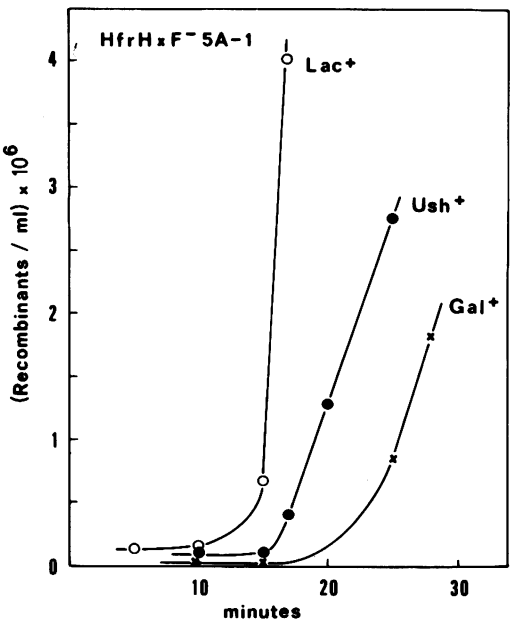


FIG. 1. *"Time of entry" experiment in the cross HfrH (AB259-1) × F⁻ 5A-1. Samples were removed from the mating cultures at intervals, shaken for 2 min on a Vortex mixer to separate the mating cells, and plated on the selective plates. The plates contained streptomycin (100 μg/ml) to eliminate the Hfr cells. Concentration of 5'-AMP as carbon source was 3 mM.*

Substrate specificity in the cryptic mutant. 5'-Nucleotidase and 3'-nucleotidase can cleave, respectively, other 5'- or 3'- nucleotides (4, 5, 21). We have compared the substrate specificity of nucleotide cleavage in strain 3-4 to its parental strain by using whole cells and cell extracts (Table 7). Although all four 5'- and 3'-nucleo-

tides tested are cleaved, presumably by the corresponding enzyme, strain 3-4 is cryptic for only three of them. It is not cryptic for cytidine monophosphate (CMP), in particular 3'-CMP.

Effect of EDTA. Ethylenediaminetetraacetic acid (EDTA) is known to increase the permeability of cells to metabolites which can usually not enter the cells (16, 29), and to release a portion of the surface lipopolysaccharides (17). We have tried the effect of EDTA on nucleotidase expression in whole cells of the cryptic strain (Table 8). Like the internal β-galactosidase, the enzymatic activity of both nucleotidases becomes significantly increased in the EDTA-treated cells. In a similar experiment (data not shown), we found that this increased activity remains bound to the cells and is not released into the medium. EDTA can, therefore, partially expose the enzyme to the substrate in the cryptic strain.

TABLE 5. *Analysis of Ush⁺ recombinants in the cross Hfr AT2455-1 × F⁻ 5A-1*[a]

Unselected marker	Percent unselected marker among Ush⁺Str^r recombinants
Lac⁺	88.6
Tsx-s	95.4
Gal⁺	34
His⁺	2.2

[a] The conjugants were mated for 60 min and plated on 5'-AMP as carbon source. The Ush⁺ Str^r recombinants were isolated, purified, and tested for segregation of unselected markers.

[b] Test of 44 purified colonies.

Release of enzyme by spheroplasts and by osmotic shock. Since one possible explanation of crypticity is that there is an alteration in localization of the enzymes which have become cryptic (see reference 33 and Introduction), we have determined whether they are still periplasmic. Indeed, it was remotely possible that the enzymes are no longer secreted. Periplasmic enzymes are released into the medium when the cells are converted to spheroplasts or subjected to an osmotic shock (12, 13); some enzymes are released only by the latter treatment and not by the former (4). The data in Table 9 show that when cells from strains AB1157-1 and 3-4 are converted to spheroplasts, using lysozyme and EDTA, both nucleotidases are released and to a comparable extent. In contrast, β-galactosidase remained within the spheroplasts. Both nucleotidases are likewise released as a result of an osmotic shock treatment (data not shown). The cryptic mutant of Ward and Glaser also releases its 5'-nucleotidase upon osmotic shock treatment (33).

Crypticity of other periplasmic enzymes. We tested two other monoesterases, alkaline phosphatase and acid hexose phosphatase, both of which are periplasmic enzymes (Table 10). Alkaline phosphatase, which is derepressed in a

TABLE 6. *Analysis of Crp⁺ recombinants in crosses with the cryptic mutant 3-4*[a]

Cross	Crp⁺ Tsx-r recombinants selected on	No. of Crp⁺ recombinants tested	Segregants among Crp⁺ recombinants (%)						
			Thr⁺	Arg⁺	Xyl⁺	Str^s	Thy⁺	His⁺	Gal⁺
P4x-1 × 3-4	5'-AMP	31	48.4	51.6	80.7	87.2		0	
P4x-1 × 3-4	3'-AMP	40	40	22.5	72.5	61		0	0
P4x-1 × 3-4/6[b]	5'-AMP	56			82	83	23	0	

[a] After 90 min of incubation, the conjugants were treated with bacteriophage T6 to eliminate the Hfr parent and were plated on AMP as carbon source to obtain Crp⁺ recombinants. These were isolated and purified, and their phenotype concerning other markers was determined.

[b] A Thy⁻ mutant of 3-4, selected on trimethoprim (31).

TABLE 7. *Substrate specificity of crypticity of nucleotidase*[a]

Strain	Determination	Specificity for:							
		5'-AMP	5'-GMP	5'-CMP	5'-UMP	3'-AMP	3'-GMP	3'-CMP	3'-UMP
AB1157-1	Whole cells	12.6	10.0	7.2	5.4	1.42	2.21	2.33	1.38
	Cell extract	20.0	15.3	9.6	5.7	1.96	2.11	2.32	1.50
3-4	Whole cells	0.4	1.0	4.4	0.5	0.04	0.09	1.83	0.05
	Cell extract	20.1	14.3	9.6	4.9	2.39	2.38	2.27	1.14

[a] Concentration of substrates was 0.33 mM; GMP, guanosine monophosphate; CMP, cytidine monophosphate; UMP, uridine monophosphate.

TABLE 8. *Effect of EDTA on crypticity in strain 3-4*[a]

Treatment	Enzyme units		
	5′-Nucleotidase	3′-Nucleotidase	β-Galactosidase
Whole cells	0.74 (4.6)	0.08 (3.3)	0.26 (8.6)
Whole cells + EDTA	4.1 (25.8)	0.95 (39.9)	1.84 (60.1)
Extract	15.9 (100)	2.38 (100.0)	3.02 (100.0)
Extract + EDTA	13.6	2.48	2.97

[a] A sample of the washed cell suspension was treated with 2×10^{-4} M EDTA (under full aeration), and 2 min later it was assayed for enzyme activity. Values are expressed as enzyme units. Figures in parentheses give percent activity.

TABLE 9. *Release of enzymes by spheroplasts*[a]

Determination	Treatment	AB1157-1 (wild-type)			3-4 (cryptic)		
		5′-Nucleotidase	3′-Nucleotidase	β-Galactosidase	5′-Nucleotidase	3′-Nucleotidase	β-Galactosidase[b]
Control	Supernatant	0.7 (93.7)	0.07 (1.9)	0.003 (4.3)	0 (0)	0.03 (0.9)	0.085 (28.8)
	Cells	4.9	1.36	0.043	0	0.28	0.182
	Extract	19.0 (96.3)	3.54 (98.1)	0.066 (95.6)	17.5 (100)	3.16 (98.9)	0.209 (71.2)
Experimental	Supernatant	16.6 (86.9)	4.42 (91.5)	0.006 (14.6)	18.25 (83)	4.64 (85.2)	0.005 (1.2)
	Spheroplasts	0.2	0.48	0.047	2.92	0.56	0.407
	Extract	2.5 (13.1)	0.41 (8.5)	0.035 (85.4)	3.73 (17)	0.81 (14.8)	0.418 (98.8)

[a] Data are enzyme units. Figures in parentheses give percent activity.

[b] Data are with strain 5-70, another cryptic mutant (Table 3) which gave the same results with regard to nucleotidase.

TABLE 10. *Alkaline phosphatase and acid hexose phosphatase in cryptic and noncryptic strains of E. coli*[a]

Determination	Alkaline phosphatase		Acid hexose phosphatase	
	AB1157-1	3-4	AB1157-1	3-4
Whole cells	2.75 (112)	0.34 (14.6)	5.26 (108)	4.95 (69.2)
Cell extract	2.46 (100)	2.32 (100)	4.84 (100)	7.16 (100)

[a] Values are enzyme units. Values in parentheses are percent activity.

low-phosphate medium, is also affected by the mutation in strain 3-4, only 14.6% of the full activity being expressed in whole cells. Acid hexose phosphatase is partially affected by the mutation, in general agreement with the results of Ward and Glaser (33). Thus, in the *crp* mutant at least three enzyme activities are affected (3′- and 5′-nucleotidases and alkaline phosphatase) and one is not (acid hexose phosphatase). The cryptic mutant previously studied showed, in addition, no crypticity for adenosine diphosphate glucose pyrophosphatase (33); it was also not cryptic for cyclic phosphodiesterase, but this enzyme was assayed with bis-(*p*-nitrophenyl) phosphate, in contrast to 3′-AMP and 2′3′-cyclic AMP used in this study.

Taken together, these results emphasize that any hypothesis must account for the apparent nonspecific nature of the *crp* lesion.

DISCUSSION

Two general hypotheses concerning the crypticity of periplasmic enzymes in the Crp$^-$ mutants described in this paper are possible, both of which were originally considered by Ward and Glaser (31). First, an alteration in the localization of the enzymes may have occured. A gross alteration, however, is ruled out by the fact that the enzymes are still located in the periplasm, as judged by their release in normal yields by osmotic shock and by making spheroplasts.

It is not known whether periplasmic proteins are associated in any way with the cytoplasmic membrane, although there is some reason to believe that they might be. For example, if binding proteins are concerned with the transport of certain compounds (18) or with chemotaxis (11, 15), they might interact in some way with the cell membrane. If this were so, in the case of Crp$^-$ mutants, this binding may be altered such that the enzyme is no longer available to external substrate. Moreover, the enzyme could remain, or become, available to

an internal substrate only, as suggested by Ward and Glaser (33, 34). These authors found no difference, by a variety of techniques, between the enzyme from wild-type and from the cryptic mutant, and favored the notion that a binding site is altered (33). Our finding that the *crp* mutation is a single one and that it is genetically distinct from the *ush* (UDP-sugar hydrolase) locus certainly demonstrates that the Crp$^-$ phenotype is not due to genetic modification of the UDP-sugar hydrolase. This is, in any case, rendered unlikely, at least in the case of the mutant described in this paper, by the fact that more than one enzyme is affected. It is, or course, possible that an altered binding site could affect more than one enzyme.

The second hypothesis is that the permeability of the outer membrane of Crp$^-$ mutants is altered.

The finding that at least three periplasmic enzymes become cryptic in the mutants is consistent with this explanation, the implication being that the substrates of these enzymes cannot penetrate the outer membrane. Since the effect is not specific for one enzyme, there is no need to postulate the absence of a *specific* transport or facilitated diffusion mechanism in the outer membrane. The finding that treatment with EDTA, which is known to increase the permeability of the cell (17, 29), substantially abolishes crypticity may be consistent with both hypotheses. Thus, it is likely that EDTA increases the permeability of the outer membrane as well as the cytoplasmic membrane, alleviating any defect in permeability of the outer membrane. On the other hand, if in the Crp$^-$ mutants the organization of the enzymes in question is subtly altered but are able to hydrolyze an internal substrate, as discussed above, then the ability of EDTA to increase the permeability of the cytoplasmic membrane would also be expected to alleviate crypticity.

With regard to acid hexose phosphatase, towards which the Crp$^-$ mutant does not show crypticity, the reason may be related to the fact that *E. coli* possesses on uptake mechanism for glucose-6-phosphate (6, 7, 35), which was used as substrate, though in our experiment it was not induced.

Lastly, we would like to comment on the lack of crypticity towards CMP (Table 7). We can only suggest that there is another enzyme, not cryptic in strain 3-4, which degrades 5′- and 3′-CMP. However, strain 5A-1, which is Ush$^-$, also lacks 5′-nucleotidase activity when assayed with 5′-CMP as substrate, which makes this possibility less likely.

In summary, although the present results rule out certain possibilities and provide a basis for future work, we have been unable to disprove either of the hypotheses discussed above. Further work will be necessary before the lesion in these cryptic mutants can be defined with certainty.

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