Analysis of an L-Histidinol-utilizing Mutant of *Pseudomonas aeruginosa*

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SUMMARY

Transductional analysis was applied to the *Pseudomonas aeruginosa* mutant PAO14 (*hnc-1*). This mutant can utilize L-histidinol as sole source of carbon and nitrogen and has a 60-fold increased histidinol dehydrogenase (HDH) content (Dhawale, Creaser & Loper, 1972). Transductional analysis was carried out using 18 histidine-requiring mutants to see where the *hnc-1* locus maps in relation to the structural genes of histidine biosynthesis. The *hnc-1* marker cotransduced with group IV genes at 97 to 100 % and not at all with group I, which is known to be the structural gene for HDH.

The data obtained in the studies of K_m (histidinol) and K_m (NAD), and the effect of pH and temperature on the HDH activity from PAO1 and PAO14 are in full agreement with the genetic data that the *hnc-1* mutation is not in the structural gene for HDH. It is suggested that *hnc-1* may be a mutation in a regulatory gene affecting HDH synthesis in PAO14 and may map close to *his-IV* whose function in histidine biosynthesis is not known.

INTRODUCTION

Previous work on the evolution of mechanisms for utilization of histidinol as sole source of carbon and nitrogen (Dhawale, Creaser & Loper, 1972) showed that whereas *Arthrobacter histidinolovorans* produced a second, inducible enzyme system for the conversion of histidinol to histidine, *Pseudomonas aeruginosa* appeared to have developed a new control system for the increased formation of histidinol dehydrogenase (HDH).

We isolated a mutant of *P. aeruginosa* which can utilize histidinol (*hnc-1*: PAO14) as the sole source of nitrogen and carbon, and gives a 60-fold increase in total HDH activity. However, polyacrylamide gel electrophoresis was unable to separate the enzyme activity of the mutant from that obtained from the parent strain PAO1 grown on glucose plus minimal medium. The 'mutant' enzyme is inducible by histidinol and its synthesis is repressed by the catabolites of histidinol and histidine. It has been suggested that HDH produced by the mutant strain PAO14 is the same as that synthesized by the wild-type strain (PAO1) grown on mineral salts, glucose and NH_4NO_3 . To test this hypothesis further, two approaches were used. Firstly, a genetic analysis was carried out using histidine-requiring mutants in order to relate the *hnc-1* mutation to other genes of histidine biosynthesis in this bacterium. Secondly, the kinetic properties of the enzymes, which included K_m (histidinol), K_m (NAD), optimum pH and optimum temperature of the enzyme reaction were studied.

There appear to be at least five groups of genes involved in histidine biosynthesis in *P.*

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aeruginosa (Mee & Lee, 1967) which are reported to be unlinked (Fargie & Holloway, 1965). Mee & Lee (1969) proposed a map order for group I genes, and phenotypes of the alleles have been examined in relation to the map order.

METHODS

Media. All percentages are w/v. Nutrient broth (NYB): Difco nutrient broth, 0.8 %; Difco yeast extract, 0.5 %. Nutrient agar (NA): Oxoid blood agar base, 4 %; Oxoid yeast extract, 0.5 %. Layer agar (LA) for phage assay: NYB plus 0.8 % Ionagar No. 2 (Oxoid). Minimal medium (MM) was that of Vogel & Bonner (1956) solidified with 1 % Ionagar No. 2 (Oxoid). Supplemented minimal media (SMM): appropriate sterile solutions (sterilized by filtering through a 0.45 μ m Millipore filter) of D- or L-amino acids were added to MM to give a final concentration of 100 μ g/ml, except isoleucine and valine (ilv), which were added to give final concentrations of 50 and 100 μ g/ml, respectively, and solidified with 1 % Ionagar No. 2 (Oxoid). *Pseudomonas* mineral medium (PMM): K_2HPO_4 , 0.15 %; KH_2PO_4 , 0.05 %; $MgSO_4 \cdot 7H_2O$, 0.02 % (Tabor & Hayaishi, 1952). Histidinol medium (HM): PMM supplemented with L-histidinol (solution sterilized separately by filtering through a 0.45 μ m Millipore filter) at a concentration of 0.5 to 1.0 mg/ml, and solidified with 1 % Ionagar No. 2 (Oxoid) for plates.

Bacterial strains. *Pseudomonas aeruginosa* strain PAO14 (*hnc-1*) was derived from strain PAO1 after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) (Dhawale *et al.* 1972). Other strains are listed in Table 1.

Bacteriophage strain. Transducing phage G101 (Holloway, Krishnapillai & Stanisich, 1971) contains double-stranded DNA with a molecular weight of 38×10^6 daltons. G101 gives more cotransduction than transducing phage F116 (V. Krishnapillai, personal communication). Phage G101 was propagated on strain PAO14 by the agar layer method, sterilized by filtration through a 0.45 μ m Millipore filter, checked for bacterial count, stored sterile at 4 °C and used within a month.

Transduction experiment. Preparations of the transducing phage G101 were adjusted to a titre of 10^9 plaque-forming units (p.f.u.)/ml with TMM buffer (tris, 0.121 %; sodium chloride, 0.877 %; $MgSO_4 \cdot 7H_2O$, 0.246 %; adjusted to pH 7.4 with 1 M-HCl, autoclaved at 15 lb/in² for 20 min). The recipient strains (Table 1) were grown overnight in 10 ml NYB at 37 °C. Exponential-phase cultures were obtained by inoculating 1 ml of overnight-grown cultures into 30 ml of prewarmed NYB and aerating at 37 °C for 2.5 to 3 h. Samples (10 ml) were centrifuged in a bench centrifuge for 15 min and the cell pellet resuspended in 2 ml sterilized saline (0.14 M-NaCl). An equal volume of donor phage preparation was added to the suspension and the mixture incubated at 37 °C for 20 min. After absorption, the mixture was centrifuged and the pellet resuspended to the same volume in saline. Samples (0.2 ml) were then plated on to MM or SMM by spreading with a glass spreader. The plates were incubated at 37 °C for 48 h before counting the transductants.

Characterization of transductants. Transductants were partially purified by spotting the colonies on to a plate of the same medium on which the transduction was performed. After overnight growth at 37 °C, this was used as the master plate in replicating on to different supplemented medium, NA, MM or HM plates. Cotransduction of the *hnc-1* marker was determined by replicating on to HM (supplemented with the required amino acid) plates. Replica plates were incubated at 37 °C for 48 h before the segregation of non-selected marker or selected marker was determined. Maleness of the strain was determined (PAO1302 and GMA130) using NA plates supplemented with 40 μ g $HgCl_2$ /ml (Holloway *et al.* 1971). Trans-

Table 1. *Pseudomonas aeruginosa* strains used

Strain*	Markers†	Description
PAOI		Wild type
PAO7	<i>his-1, met-28</i>	Methionine-, histidine-requiring mutant
PAO14	<i>hnc-1</i>	L-Histidinol-utilizing mutant isolated by treatment of NTG on PAOI
PAO27	<i>ser-3, his-33</i>	Serine-, histidine-requiring mutant
PAO67	<i>his-67, ese-2</i>	Histidine-requiring, phage E 79-resistant mutant
PAO73	<i>his-70, ese-2</i>	Histidine-requiring, phage E 79-resistant mutant
PAO246	<i>his-3, met-28, trp-6, lys-56</i>	Histidine-, methionine-, tryptophan- and lysine-requiring mutant
PAOI302	<i>his-161, FP⁺</i>	Histidine-requiring mutant, male strain
PAOI651	<i>his-171, met-28, ilv 282</i>	Histidine-, methionine- and isoleucine-valine-requiring mutant
M2355	<i>his-5037</i>	Histidine-requiring mutant
M2356	<i>his-5039</i>	Histidine-requiring mutant
M2357	<i>his-5044</i>	Histidine-requiring mutant
M2360	<i>his-5065</i>	Histidine-requiring mutant
GMAO34	<i>his-5034, ade-5034</i>	Histidine- and adenine-requiring mutant
GMAO35	<i>his-5035</i>	Histidine-requiring mutant
GMAI22	<i>his-69</i>	Histidine-requiring mutant
GMAI30	<i>his-64, trp-4, FP⁺</i>	Histidine- and tryptophan-requiring mutant, male strain
GMAI47	<i>his-23, ade-66</i>	Histidine- and adenine-requiring mutant
GMAI56	<i>his-49, ade-66</i>	Histidine- and adenine-requiring mutant
GMAI59	<i>his-9, cys-5242</i>	Histidine- and cysteine-requiring mutant

* Strains prefixed PAO were obtained from Professor B. W. Holloway, strains prefixed M from Dr B. J. Mee, and strains prefixed GMA from Dr A. H. C. Kung.

† This nomenclature follows Demerec *et al.* (1966).

ductants were tested for immunity to the lysogenizing phage G101 by either streaking against phage on NA or by replicating on to a NA plate spread with 0.1 ml phage suspension.

Preparation of crude extracts of Pseudomonas aeruginosa strains PAOI and PAO14. *Pseudomonas aeruginosa* strain PAOI was grown in 1 l PMM supplemented with 1 % glucose and 0.2 % NH_4NO_3 . The medium was shaken at 37 °C after inoculating with a colony of culture grown overnight on a NA plate. The strain PAO14 was grown in 1 l PMM supplemented with 0.05 % histidinol. The medium was shaken at 37 °C after inoculating with a colony grown overnight on a HM plate. The cultures were harvested when they reached stationary phase. Cells were collected by centrifugation at 13000 g for 20 min. Cells were suspended in 10 ml tris-HCl buffer (pH 9.1, 0.05 M) containing 0.1 % 2-mercaptoethanol, and sonicated with ice-cooling for 5 min each in an MSE 100 W disintegrator. The resultant extracts were used directly for enzyme assays.

Partial purification of histidinol dehydrogenase. The method for partial purification of HDH from various strains of *P. aeruginosa* was as described by Dhawale *et al.* (1972).

Estimation of protein. Protein was estimated by the method of Lowry *et al.* (1951) using bovine albumin fraction V (Sigma) as standard.

Enzyme assays. L-Histidinol dehydrogenase was assayed by NAD reduction, measuring the rate of increase in E_{340} at 37 °C (Creaser, Bennett & Drysdale, 1967). A spectrophotometer cuvette (10 mm light path) contained 0.7 ml of glycine-NaOH buffer, pH 9.8 (3.78 g glycine adjusted to pH 9.8 with 1 M-Na OH and made up to 1 l) containing 0.1 % (v/v) 2-mercaptoethanol, 0.1 ml aqueous NAD (1 μmol) and 0.1 ml of the enzyme solution under test.

The mixture was incubated at 37 °C in the cell for 2 min and then 0.1 ml (0.2 μ mol) of L-histidinol dihydrochloride solution was added to initiate the reaction. NAD reduction was then measured. Enzyme activities are expressed in nmoles NAD reduced per minute (μ).

Phosphoribosyl-ATP (PR-ATP) synthetase activity can be measured as described by Voll, Apella & Martin (1967), which is based upon the measurement of the relatively high extinction coefficient of PR-ATP at 290 nm. A 10 mm spectrophotometer cuvette contained 0.15 ml buffer salt solution (0.2 M-tris-HCl pH 8.5, 0.02 M-MgCl₂, 0.3 M-KCl), 10 μ l pyrophosphatase (0.1 mg/ml, Sigma), 20 μ l ATP (0.1 M), enzyme solution, and water to make 0.95 ml. The reaction was started by adding 50 μ l phosphoribosyl pyrophosphate (PRPP) (6.6 mM in 20 mM-EDTA at pH 7). The preparation was mixed and the increase in extinction at 290 nm and 37 °C was recorded. One unit of activity corresponds to the formation of 0.00167 μ mol PR-ATP/min – a ΔE_{290} of 0.05/min.

Imidazole glycerol phosphate (IGP) dehydratase activity was determined by the method of Ames (1957). Imidazole acetol phosphate (IAP) formed from IGP absorbs strongly in the ultraviolet and was measured at 290 nm after enolization. A reaction mixture contains 0.6 ml of the assay mixture (5 ml of 0.1 M-triethanolamine pH 8.1, 30 μ l of 14.3 M-3-mercaptoethanol, 10 μ l of 0.1 M-MnCl₂) and 20 to 100 μ l enzyme. The reaction is started with 20 μ l of IGP (0.1 M, synthesized according to the procedure of Ames, 1957). A blank with water in place of IGP was carried through the procedure. The mixture was incubated at 37 °C for 30 min and was stopped by the addition of 1.38 ml of 1.43 M-NaOH. The mixture was re-incubated at 37 °C for 30 min to accomplish the enolization of IAP formed. A blank measured the E_{290} without enzyme. One unit of enzyme forms 1 μ mol IAP/h, resulting in a ΔE_{290} of 5.1.

Histidinol phosphate (HP) phosphatase assay was based on the determination of inorganic phosphate released in the reaction, according to Ames, Hartman & Jacob (1963). The enzyme (0.1 ml) was added to the buffer (triethanolamine-HCl; 0.1 M, pH 7.5) to a final volume of 0.29 ml. A blank without enzyme was brought to 0.3 ml. The reaction was initiated with 10 μ l of histidinol phosphate (50 mM; Calbiochem), and the assay mixture incubated at 37 °C for 15 min. At the end of 15 min, 0.7 ml of colour-developing mixture was added (1 part of 10 % ascorbic acid plus 6 parts of 0.42 % ammonium molybdate in 0.5 M-phosphate-free H₂SO₄). The colour was developed by incubating the mixture at 45 °C for 20 min. The precipitate was discarded following centrifugation for 10 min in a bench centrifuge. The colour of the resulting supernatant was measured at 820 nm against the blank. Values were corrected by assaying a control (minus enzyme, plus histidinol phosphate). One unit of activity lead to the formation of 1 unit of extinction in 15 min at 820 nm, or one unit of enzyme hydrolysed 0.0385 μ mol histidinol phosphate/15 min at 37 °C.

IAP-glutamate aminotransferase activity was measured by the method of Martin & Goldberger (1967), which measures the extinction of imidazole acetol phosphate when it is enolized in strong alkali. The reaction was run in the reverse direction so that formation of IAP from histidinol phosphate could be measured at 280 nm. The reaction mixture contained 0.4 ml buffer (triethanolamine-HCl; 0.2 M, pH 8.6), 10 μ l 2-ketoglutarate (0.2 M, pH 6.0) and enzyme (20 to 100 μ l). The reaction was started by the addition of 10 μ l of histidinol phosphate (50 mM) and the mixture incubated at 37 °C for 15 min. A blank with water was carried through the assay procedure. The reaction and blank were stopped by the addition of 1.4 ml of 1.43 M-NaOH and re-incubated at 45 °C for 20 min to enolize the IAP formed. The resulting E_{280} was measured against a blank. One unit of enzyme activity yielded an extinction of 1, or converted 0.188 μ mol histidinol phosphate to IAP at 37 °C every 15 min.

Table 2. Transductional analysis of 18 histidine-requiring mutants of *P. aeruginosa*

Transductants were selected only for His⁺. Transducing donor phage G101 was propagated on strain PA014 (*hnc-1*).

Recipient strain	Group no.	No. of transductants/ 10 ⁹ donor phage	No. of transductants scored	No. of transductants carrying <i>hnc-1</i> marker
PA07	I	88	100	0
PA027	IV	130	100	100
PA067	II	148	100	0
PA073	IV	2	44	43
PA0246	IV	25	164	164
PA01302	?	28	100	0
PA01651	?	0	0	0
M2356	I	2	32	0
M2357	II	24	86	0
M2360	III	5	121	0
M2355	V	0	0	0
GMA130	IV	95	100	100
GMA147	I	15	62	0
GMA156	I	12	84	0
GMA034	I	17	65	0
GMA035	V	5	40	0
GMA122	II	21	84	0
GMA159	I	13	65	0

RESULTS AND DISCUSSION

Of 18 histidine-requiring mutants of *P. aeruginosa* tested, all were negative for *hnc-1* marker (i.e. they do not show utilization of histidinol as sole source of nitrogen and carbon). However, for the growth of these His⁻ mutants in PMM + glucose + NH₄NO₃ medium, histidine (growth requirement) could be replaced by histidinol, which indicates that inability to use histidinol as sole source of nitrogen and carbon cannot be due to impermeability.

Transductional analysis of these 18 His⁻ mutants which fall into five groups, using PA014 (*hnc-1*) as donor and selecting for His⁺ transductants and then scoring for *hnc-1* marker, showed that this marker is cotransducible with *his* alleles of group IV at about 97 to 100 % (Table 2). Although all these mutants adsorb the transducing phage G101 at similar levels and form plaques at the same efficiency, they gave different frequencies of His⁺ transductants.

When the experiment was repeated using the group IV strain PA0246 as recipient but first selecting for *hnc-1*, 28 transductants were obtained: all 28 were *his-3*⁻. From the previous experiment we can infer that all 28 should be *his-3*⁺, cotransduction being 100 % with this recipient. The simplest explanation is that the *his-3*⁺ gene was cotransduced but that growth on histidinol had in some way resulted in its not being expressed. Further transductional work on the 28 isolates was not feasible as they were all G101 lysogens.

Kinetic properties of HDH in *P. aeruginosa*

Double reciprocal plots of the concentration of the substrate (histidinol) or co-factor (NAD), and the velocity of the reaction catalysed by HDH were employed to calculate the Michelis constants. They showed that *K_m* (histidinol) and *K_m* (NAD) for HDH produced by the mutant strain PA014 are the same as that for HDH produced by the wild-type strain PA01 (Fig. 1*a, b*). The plots of HDH activity versus temperature (Fig. 2*a*) and pH (Fig. 2*b*)

Fig. 1. Lineweaver-Burk plot to determine (a) K_m (histidinol) and (b) K_m (NAD) of histidinol dehydrogenase in *P. aeruginosa* strains PAO1 and PAO14. Δ , Strain PAO1; $\circ$, strain PAO14. Activity was measured in a 1 ml total volume of glycine-NaOH buffer, pH 9.8, containing 0.1 % 2-mercaptoethanol. For the determination of K_m , either (a) 1 μ mol NAD, or (b) 0.2 μ mol histidinol, each in 0.1 ml, plus 0.1 ml enzyme solution were added and the mixture incubated in the spectrophotometer cuvette for 2 min before initiation of the reaction by the addition of (a) 8 to 80 nmol histidinol solution, or (b) 4 to 40 nmol NAD solution. The initial velocity v is expressed in milliunits.

Fig. 2. Effect of (a) temperature and (b) pH on the activity of histidinol dehydrogenase in *P. aeruginosa* strains PAO1 and PAO14. Δ , Strain PAO1; $\circ$, strain PAO14. Activities were measured in 1 ml total volume. (a) A mixture of 0.75 ml of glycine-NaOH buffer, pH 9.8, 0.05 ml enzyme solution and 0.1 ml NAD solution (1 μ mol) was incubated at the required temperature for 5 min in the spectrophotometer cuvette. The reaction was initiated by the addition of 0.1 ml of L-histidinol solution (0.2 μ mol). NAD reduction was then measured. Temperature was regulated by means of a water circulator. (b) All buffers were 0.05 M, and contained 0.1 % 2-mercaptoethanol. Buffers of pH values 8.6 to 10.4 were glycine-NaOH, and 7.2 to 8.2 were tris-HCl; buffer of pH 6.8 was sodium-potassium phosphate.

indicate that the HDH produced by both the strains (PAO1 and PAO14) express maximal activity at a pH value of 9.6 and at a temperature of 46 °C (Table 3).

Effect of hnc-1 mutation on activities of other enzymes of histidine biosynthesis

Having observed a marked increase in HDH activity in *P. aeruginosa* strain PAO14, it is of interest to know the effect of *hnc-1* mutation on the other enzymes of histidine biosynthesis. Enzyme assays were carried out using crude extracts of strain PAO1 grown on PMM + glucose + NH_4NO_3 and of PAO14 grown on histidinol medium. The results (Table 4) indicate a

Table 3. Kinetic properties of partially purified HDH produced by *P. aeruginosa* strains PAOI and PAOI4

K_m (histidinol)	3.9×10^{-5} M
K_m (NAD)	2.3×10^{-4} M
Optimum pH	9.6
Optimum temperature	46 °C

Table 4. Specific activities of enzymes of histidine biosynthesis in strains of *P. aeruginosa*

Enzymes tested	Specific activity (u/mg protein)	
	PAOI	PAOI4
Phosphoribosyl-ATP synthetase	ND	ND
IGP dehydratase	0.61	0.52
HP phosphatase	0.11	0.15
IAP glutamate aminotransferase	0.18	0.10
Histidinol dehydrogenase*	0.054	8.6

ND, Not detectable. * Activity expressed as mu/mg protein.

Table 5. HDH specific activity in histidine requiring mutants of *P. aeruginosa*

Group	HDH specific activity (mu/mg protein)
I	ND
II	0.044
III	ND
IV	0.156
V	1.7

ND, Not detectable.

slight decrease in IGP dehydratase activity and increase in HP phosphatase activity in strain PAOI4 which were not significant when compared with the activities in strain PAOI. Activity of the enzyme IAP glutamate aminotransferase in strain PAOI was about twice that of strain PAOI4. In both the strains PR-ATP synthetase activity was undetectable. These data suggest that the *hnc-1* mutation does not cause any significant alteration in the levels of enzymes tested, other than HDH.

In *P. aeruginosa*, histidine groups II, IV and V are suggested as not being involved in the control of HDH synthesis. This is based on the fact that mutants from these groups show the presence of HDH activity with varying levels (see Table 5). As group III mutants can take up histidinol when supplied in place of histidine as a growth requirement with MM, this suggests that the group III mutant is not devoid of HDH activity, but cells synthesize the enzyme to such a small extent that it was not detectable by the assay system. On comparison of the HDH enzyme produced by strain PAOI4 grown on HM, and that produced by wild-type strain PAOI grown on PMM + glucose + NH_4NO_3 , these show similar enzyme kinetics, similar optimum pH and temperature for expression of maximal activity, and are indistinguishable electrophoretically (Dhawale *et al.* 1972). If we assume that mutation or mutations in a structural gene(s) must affect the nature of the enzyme produced by the gene(s), then one must postulate that on the basis of information available, the *hnc-1* mu-

tation is not in the structural gene but must be in a second gene. Further support for this postulation comes from the transductional analysis of *his* mutants of *P. aeruginosa* (Table 2). The *hnc-1* mutation has shown a clear relationship to group IV genes. Group I is considered to be a structural gene for HDH synthesis in *P. aeruginosa* (Mee & Lee, 1969), and therefore the structural gene and hypothesized second gene map at quite separate regions of the chromosome.

The higher induced level of HDH enzyme in this mutant (PAO14) can be explained in that the mutant may be accumulating a higher cell concentration of effector molecules (histidinol), which may be due to the effect of altered membrane permeability. Existence of two high-affinity transport systems in *P. aeruginosa* which recognize tryptophan, phenylalanine and tyrosine, has been reported by Kay & Gronlund (1971). In strain PAO1 an active transport system may be present only for histidine but not for histidinol. Mutants which cannot make histidine but are not blocked at the HDH step are presumed to be able to take up histidinol by a general low-efficiency permease or by passive diffusion. The *hnc-1* mutation may have produced an altered permease system specific for histidinol. We are investigating this possibility.

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