Summary

Mutants of Bacillus subtilis deficient in urocanase or in imidazolonepropionate hydrolase were isolated. These mutants are unable to use L-histidine as source of carbon or nitrogen for growth. The enzymes are thus essential for the metabolism of histidine. The structural genes specifying these enzymes are closely linked to those specifying the other two enzymes essential for histidine degradation, histidase, and formiminoglutamate hydrolase. The response of these enzymes to induction and catabolite repression is specified by a region of the chromosome closely linked to one end of the cluster of structural genes. The gene cluster presumably constitutes an operon.

The induction by L-histidine and repression by catabolites of three of the four enzymes required for the degradation of L-histidine by Bacillus subtilis have been described in earlier papers (1-3). The enzyme catalyzing the third step in the pathway of histidine degradation, 4-imidazolone-5-propionate hydrolase, had not been previously investigated. The present paper shows that the formation of this enzyme is controlled in the same manner as that of the others. Furthermore, the isolation of mutants lacking IPA* hydrolase and of mutants lacking urocanase permitted the complete mapping of the structural genes and regulatory genes responsible for the controlled degradation of histidine. The results suggest that this cluster of genes constitutes an operon.

Experimental Procedure

Chemicals—In addition to the chemicals described in the preceding paper (3), N-methyl-N-nitrosoguanidine was purchased from Aldrich Chemical Company, and streptomycin sulfate from Squibb. IPA, a gift of Dr. Peter Scotto, was prepared and purified according to the following procedure (4). Strain N-5 of Aerobacter aerogenes (5), which is deficient in IPA hydrolase, was grown in a medium containing succinic acid as source of carbon and 0.1% imidazolepropionic acid as inducer of the histidine-degrading enzymes (6). The cells from a 600-ml culture were harvested and sonically disrupted as described previously (7). A 5-ml portion of the extract was added to a solution containing 3.0 g of L-histidine in 100 ml of 0.3 M Tris-HCl buffer, pH 9.0. The mixture was incubated at 37° for 3 hours. At this stage, approximately one-half of the histidine had been converted to urocanic acid and approximately 5% to IPA. The pH was adjusted to 7.4 by the addition of 2 N HCl; the air was evacuated from the reaction flask and replaced by nitrogen. A 10-ml portion of the cell extract was added to the reaction mixture. The atmosphere of the reaction flask was again replaced by nitrogen. The enzymatic reaction was allowed to proceed at 37° for a period of 90 min. It was arrested by the addition of sufficient 2 N HCl to lower the pH to 1.5. The precipitated protein was removed by centrifugation at 20,000 × g for 30 min. The supernatant was placed on a column (2 × 20 cm) of AG 50-W* Dowex (200 to 400 mesh) wrapped in aluminum foil in a dark room at 4°. The column was then washed with several bed volumes of water and of 0.5 N HCl. The IPA was eluted with 2 N HCl. Fractions of 10 ml were collected and examined for ultraviolet extinction at 234 and 260 mμ. The IPA, with a peak extinction at 234 mμ at a pH of 1 (molar extinction 3500), was found in tubes 20 through 60. The contents of the tubes were pooled and evaporated to dryness at room temperature in a vacuum. The residue was dried over P2O5 and NaOH and stored at -20°. The yield, estimated spectrophotometrically, based on histidine was 30%. The material had the characteristic absorption spectrum of IPA (4).

Enzyme Assays—The cultivation of the bacteria has been described (2). To prepare extracts for the assay of the enzymes, the cells from 40 to 50 ml of a culture in the exponential phase of growth were collected by centrifugation, washed with 0.02 M Tris-HCl buffer, pH 8.0, and suspended in 0.95 ml of this buffer. A 0.05-ml portion of a solution containing 4 mg of lysozyme per ml was added and the suspension was incubated for 10 to 20 min at 37°. The mixture was then cooled in an ice bath and the cells were treated at 1.2 to 1.5 atm in an MSE sonicator (Instrumentation Associates) for 1 to 2 min. A 0.1 ml portion of a 10% solution of streptomycin sulfate was added to the product of sonic disruption of cells to precipitate nucleic acids. The mixture was kept for 30 min in an ice bath and was then subjected to centrifugation at 22,000 × g at 4° for 20 min. The supernatant was used for the enzyme assays. The concentration of protein in this extract was determined colorimetrically (8).

Histidase (1), urocanase (2), and FGA hydrolase (3) were measured by procedures described elsewhere.

IPA hydrolase was assayed spectrophotometrically by following...
the rate of disappearance in the absence of oxygen of its extinction at 260 με (4, 9). A volume of extract containing approximately 1 mg of protein was used. Sufficient 0.1 M potassium phosphate buffer, pH 7.4, to make the sum of the volumes of extract and buffer 2.96 ml was placed in the main compartment of a Thunberg tube fused to a quartz cuvette, and 0.05 ml of 0.015 M IPA in 0.2 N HCl was placed in the top compartment. The tube was evacuated, opened, and the cell extract was added to the main compartment. The tube was then thoroughly evacuated and incubated at 37° for 10 min. The substrate was mixed with the contents of the main compartment and the decrease in extinction at 260 με was recorded in a Gilford recording spectrophotometer at 37°. The reaction was linear until approximately 15% of the substrate had disappeared. Because of the instability of the substrate it was necessary to subtract the decrease in extinction measured in a similar Thunberg tube containing 0.5 to 2 mg of crystalline serum albumin in place of cell extract.

**Bacteria**—The strains are listed in Table I. The cultivation of the bacteria and the methods used for their transformation have been described (2).

Mutants unable to form urocanase were isolated from cells of the wild strain SH treated with N-methyl-N-nitro-nitrosoguanidine (10). The cells in which mutagenesis had been induced were plated, at approximately 200 colonies per plate, on agar containing citrate and no nitrogen source other than histidine. Approximately 90 colonies of a total of 5000 showed very poor growth on that medium. Those failing to grow on plates containing glutamate were eliminated and the remainder were grown on tryptone agar plates supplemented with 0.1% histidine and tested for the presence of histidase and urocanase activity. One of the strains, SH-7, was chosen for further study.

A mutant defective in IPA hydrolase was obtained by mutagenesis with N-methyl-N-nitro-nitrosoguanidine (10) of strain SH-11, which forms the other enzymes of histidine degradation constitutively and without sensitivity to catabolite repression (2). In this case the cells in which mutagenesis had been induced were spread on agar plates containing glucose and ammonium sulfate to give 200 colonies per plate. These were replicated to glucose-histidine agar plates. The colonies which failed to reproduce on these plates were tested for histidase and urocanase (11). Of a total of 7000 colonies, 25 failed to grow on the glucose-histidine plates, but possessed both histidase and urocanase. These strains were cultivated in liquid medium containing glucose as major source of carbon and induced with histidine; extracts were prepared and tested for IPA hydrolase and FGA hydrolase. One was found to be deficient in IPA hydrolase and was designated SH-8. The other mutants lacked FGA hydrolase. Two of them, strains SH-111 and SH-112, were shown to produce material that immunologically cross-reacts with FGA hydrolase (3).

**RESULTS**

**Role and Control of Urocanase and 4-Imidazolone-5-propionate Hydrolase**—We examined the levels of the four enzymes of histidine degradation in a variety of mutants. The results, summarized in Table II, show that the wild strain SH produces the four enzymes when grown in a histidine-containing medium. Strains

**Table I**

**Bacterial strains**

The symbols used are: ery-r, resistant to 1 μg per ml of erythromycin; hut H, loss of histidase activity; hut I, loss of IPA hydrolase activity; hut G, loss of FGA hydrolase activity; hut P, pleiotropic loss of the four enzymes of histidine degradation; hut R, insensitivity of the histidine-degrading enzymes to catabolite repression; hut C, constitutive histidine-degrading enzymes. A mutant genotype is indicated by an isolation number or in the general case by a minus sign. In addition to the strains listed, erythromycin-resistant derivatives of most of the mutants were constructed by the introduction of the ery-r mutation of strain SH-E. These derivatives are designated by the original strain number followed by the letter "E."

| Strain | Relevant genetic constitution | Derivation |
|--------|-------------------------------|------------|
| SH..... | wild type                     | Reference 2 |
| SH-E... | ery-r                         | Reference 2 |
| SH-3...
| hut H1                          | Reference 2 |
| SH-5...
| hut P1                          | Reference 2 |
| SH-7...
| hut U1                          | N-Methyl-N-nitro-
|      | guanidine mutagenesis of SH     |            |
| SH-8...
| hut R1, C1, I1                 | N-Methyl-N-nitro-
|      | guanidine mutagenesis of SH     |            |
| SH-11... | hut R1, C1                   | Reference 2 |
| SH-12... | hut R1, G1                    | Reference 2 |
| SH-111... | hut R1, C1, G2              | N-Methyl-N-nitro-
|      | guanidine mutagenesis of SH    |            |
| SB25-3... | hut H1                      | Reference 2 |
| SB25-113... | hut R1, C1, H3         | Reference 2 |

**Table II**

**Enzyme levels in wild strain SH and in mutants**

Cultures were grown from a small inoculum in minimal media containing glutamate or glucose as source of carbon. Histidine was present, where indicated, for the entire growth period in the case of strains SH, SH-11, and SH-5, but for only the last two generations of growth for strains SH-7 and SH-8. The cells were harvested during the exponential phase of growth. The enzyme activities in the extracts were measured, specific activity being expressed as millimicromoles of substrate used or of product formed per min per mg of protein.

| Carbon source | Histidine addition | Specific activity |
|---------------|--------------------|-------------------|
|               | Histidase | Urocanase | IPA hydrolase | FGA hydrolase |
| Strain SH     | Glutamate         | -         | 0.2         | <0.05 <0.05 | 1     |
|               | Glutamate         | +         | 94          | 2.4 | 0.68 | 120   |
|               | Glucose           | -         | 0.15        | <0.05 <0.05 | 1     |
|               | Glucose           | +         | 14.2        | 0.17 <0.05 | 22    |
| Strain SH-11  | Glutamate         | -         | 124         | 5.7 | 2.2  | 250   |
|               | Glutamate         | +         | 533         | 8.2 | 5.5  | 340   |
|               | Glucose           | -         | 49          | 2.8 | 1.6  | 80    |
|               | Glucose           | +         | 204         | 6.7 | 5.9  | 230   |
| Strain SH-5   | Glutamate         | +         | 2.5         | <0.05 <0.05 | 1     |
| Strain SH-11  | Glutamate         | +         | 80          | <0.05 | 0.40 | 70    |
|               | Glutamate         | +         | 105         | 4.5  | <0.05 | 110   |
SH-7 and SH-8, selected for their inability to use L-histidine as a source of nitrogen, are unable to produce, respectively, urocanase and IPA hydrolase. Thus, these two enzymes play essential roles in the conversion of histidine to glutamate and formamide. It has been shown previously that in the wild strain SH, histidase, urocanase, and FGA hydrolase are subject to induction by histidine and to repression by catabolites derived from glucose (2). It can be seen that the same controls affect IPA hydrolase. Moreover, mutations to constitutive synthesis and insensitivity to catabolite repression in strain SH-11 affect the control of all four enzymes. Finally, strain SH-5, previously shown to have lost the ability to produce histidase, urocanase, and FGA hydrolase, could not be used in this manner because of its low competence.

A series of crosses between mutants unable to utilize histidine was carried out. Recombinants capable of growth on histidine were selected, as described previously (2). In all of these crosses the recipient was sensitive to erythromycin and the donor resistant. The gene determining response to erythromycin is not linked to the hut genes and was used as reference marker in order to normalize variations in competence of the recipients in the independent experiments (2). This was done by determining in each cross the number of erythromycin-resistant (ery-r) transformants and for each recipient the number of hut+ transformants when DNA from the wild type, strain SH, was used. The results are expressed as the ratio of hut+ over ery-r transformants with mutant DNA divided by the ratio of hut+ over ery-r transformants with wild type DNA.

The results, summarized in Table III, show that hut H1 is more closely linked to hut Ul than to hut II or hut G1; hut Ul is more closely linked to hut II than to hut G1; hut II is more closely linked to hut G1 than to the other markers. Finally, hut G2, which has been shown to produce material immunologically cross-reacting with FGA hydrolase (3), is very closely linked to hut G1.

In another series of crosses the linkage of hut H1, hut Ul, and hut G1 to hut R1 was determined. The strain carrying the hut R1 mutation can produce the histidine-degrading enzymes in the presence of glucose (2). In these crosses, the DNA was derived from hut+ strains; transductants capable of using histidine as nitrogen source when citrate served as carbon source were selected and examined for their ability to use histidine as nitrogen source when glucose served as carbon source. The results show that the closeness of linkage to hut R1 declines in the order hut H1, hut Ul, hut G1 (Table IV).

The experiments presented in Tables III and IV suggest the order hut R, H, U, I, G. We carried out a series of three-factor crosses to determine the order unambiguously. In these crosses, recombinants for two markers were selected and tested for the distribution of the third marker as described previously (2). The

**Table III**

Recombination indices from two-factor selective crosses between mutants defective in histidine degradation

Transformed cultures were diluted and plated onto citrate minimal plates containing L-histidine as sole nitrogen source to score transformants with an intact histidine-degrading system of induction and repression. Moreover, mutations to constitutive synthesis and insensitivity to catabolite repression in strain SH-11 affect the control of all four enzymes. The results presented here and in an earlier report have described genetic crosses between mutants defective in histidase or FGA hydrolase or with altered control of the histidine-degrading enzymes (2). These crosses, carried out by transformation, established the close linkage of these characters. We extended this study using the newly isolated urocanase- or IPA hydrolase-deficient mutants, strains SH-7 and SH-8, as source of transforming DNA. Strain SH-7 was also used as recipient in the transformation experiments; strain SH-8 could not be used in this manner because of its low competence.

### Table III

| DNA donor | Recipient | Markers | Average recombination index |
|-----------|-----------|---------|-----------------------------|
| SH-7E     | SB22-3    | hut    | ×100                        |
| SH-3E     | SB22-3    | hut    | ×100                        |
| SH-6E     | SB22-3    | hut    | ×100                        |
| SH-12E    | SB22-3    | hut    | ×100                        |
| SH-6E     | SH-7      | hut    | ×100                        |
| SH-12E    | SH-7      | hut    | ×100                        |
| SH-3E     | SH-12     | hut    | ×100                        |
| SH-8E     | SH-12     | hut    | ×100                        |
| SH-12E    | SH-111    | hut    | ×100                        |

* From Chasin and Magasanik (2).

### Table IV

Mapping of nonselected markers

The transformants were selected for their ability to use histidine as nitrogen source with citrate as carbon source as described in Table III. They were then tested for their ability to use histidine as nitrogen source with glucose as carbon source as described previously (2).

| DNA donor | Recipient | Phenotype selected | No. of colonies of phenotype | Markers | Recombination |
|-----------|-----------|--------------------|-----------------------------|---------|---------------|
| SH 11     | R1, H+    | SH-3               | Hut R+                      | H+      | 0             | SH-11 R1-H+   |
| SH 11     | R1, U+    | SH-7               | Hut R+                      | H+      | 0             | SH-11 R1-U+   |
| SH 11     | R1, H+    | SH-12              | Hut R+                      | H+      | 52            | SH-11 R1-G1   |
| SH         | R1, G+    | SH-3               | Hut R+                      | H+      | 0             | SH-3 R1-H+   |
| SH         | R1, G+    | SH-7               | Hut R+                      | H+      | 0             | SH-7 R1-G1   |
| SH         | R1, G+    | SH-12              | Hut R+                      | H+      | 52            | SH-12 R1-G1  |

Linkage of Genes Determining Histidine Utilization (hut)—An earlier report has described genetic crosses between mutants deficient in histidase or FGA hydrolase or with altered control of the histidine-degrading enzymes (2). These crosses, carried out by transformation, established the close linkage of these characters. We extended this study using the newly isolated urocanase- or IPA hydrolase-deficient mutants, strains SH-7 and SH-8, as source of transforming DNA. Strain SH-7 was also used as recipient in the transformation experiments; strain SH-8 could not be used in this manner because of its low competence.

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The experiments presented in Tables III and IV suggest the order hut R, H, U, I, G. We carried out a series of three-factor crosses to determine the order unambiguously. In these crosses, recombinants for two markers were selected and tested for the distribution of the third marker as described previously (2). The
results of these experiments are shown in Table V. The reciprocal Crosses 1 and 2 indicate the hut H3 is located between hut RI and hut U1 and the reciprocal Crosses 3 and 4 indicate that hut U1 is located between hut RI and hut GI. Cross 5 places hut U1 between hut RI and hut H1; it excludes the possibility that hut H1 is located between hut RI and hut U1. Cross 6 indicates that hut H1 is located between hut RI and hut H1 and excludes the possibility that hut H1 is located between hut RI and hut H1. Finally, Cross 7 places hut H1 between hut Cl, known to be closely linked to hut RI (2) and hut GI. It is clear that these results are in complete agreement with the order suggested by the two-factor crosses.

**DISCUSSION**

The genetic map of the hut region of the _B. subtilis_ chromosome, established by the experiments reported earlier (2) and in this paper is shown in Fig. 1. There is no question that _hut H_ and _hut G_ are the sites of the structural genes for histidase and FGA hydrolase. In both cases mutants defective exclusively in one of these two enzymes and mapping in these regions have been shown to produce material that cross-reacts immunologically with histidase (strain SH-4, _hut H2_ (2)) or with FGA hydrolase (strain SH-111, _hut G2_ (3)). It is very likely that _hut U_ and _hut I_ are the sites of the structural genes for urocanase and IPA hydrolase, since the mutants with defects in these sites lack specifically urocanase or IPA hydrolase activity. The structural genes of the four enzymes essential for the conversion of _L-_histidine to ammonia, _L-_glutamate, and formamide appear to be located on the chromosome in the order of the sequence of the enzymatic reactions.

The group of four structural genes is closely linked to a region of the chromosome that regulates their expression (2). Mutations in this region prevent the formation of the four enzymes ( _hut P_ ), render them insensitive to catabolite repression ( _hut R_ ), or permit their synthesis in the absence of inducer ( _hut C_ ). This arrangement suggests that the genes affecting the degradation of histidine are organized as an operon. This view receives support from the results of the following paper (12), which indicates that histidase and FGA-hydrolase respond sequentially to induction. However, the definitive proof that the system is indeed an operon requires the demonstration that the state of the control gene affects only the genes in cis position. Unfortunately, so far it has not been possible to construct the merodiploid strains of _B. subtilis_ required for this demonstration.

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