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Induction of Nonpigmented Variants of *Erwinia herbicola* by Incubation at Supraoptimal Temperatures

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Several strains of *Erwinia herbicola* produce white variants at high frequency when grown in yeast beef broth at 37 C. The relationships of the variants of *E. herbicola* Y46 to the parent strain have been studied with respect to cultural, morphological, physiological and serological features. All the white variants are auxotrophic, showing a requirement for thiamine, and no instance of reversion was observed. Representative variants show reactions similar to the parent strain on treatment with both virulent and temperate phages. Possible mechanisms by which the variants could arise are indicated.

Erwinia herbicola (Geilinger) Dye (4, 11, 12) is a yellow-pigmented, small gram-negative rod which occurs widely as an epiphyte. In addition to many other sites, it can invariably be isolated from lesions of apple and pear trees suffering from the "fire-blight" disease caused by *E. amylovora* (2, 3, 15, 17), and there is some speculation that *E. herbicola* may be exerting a physiological influence on the course of the disease. Several laboratories are attempting to elucidate what role, if any, is played by *E. herbicola* in the "fire-blight" syndrome (5, 7, 15, 16).

During the course of a study of the metabolism of the β-glucoside, phloridzin, by strain Y46 of *E. herbicola*, and the involvement of the enzymes β-glucosidase and phloretin hydrolase in this degradation process (5, 6), it was observed that cultures of this organism which had been incubated at 37 C, instead of the optimal 28 to 30 C, gave rise to nonpigmented variants in relatively large numbers. The variants are stable, have an absolute requirement for thiamine not exhibited by the parent strain, and usually show modified responses to some of the physiological tests applied to them.

This paper describes the observation, and documents the characteristics of the nonpigmented, heat-induced variants, together with those of yellow derivatives of the parent strain subjected to similar treatment. A preliminary report of this work has been presented (A. K. Chatterjee and L. N. Gibbins, Proc. Can. Soc. Microbiol., June 1970).

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MATERIALS AND METHODS

Organisms and cultural conditions. The strains of *E. herbicola* used in this study are listed in Table 1. They had been previously characterized in a numerical taxonomic study of this and related species of *Erwinia* (A. K. Chatterjee, M.Sc. Thesis, Univ. of Guelph, 1968) and are maintained in the Culture Collection of the Department of Microbiology, University of Guelph. The derivatives of *E. herbicola* Y46 which are specifically mentioned in the text are listed by phenotype in Table 4.

All stock cultures were maintained on yeast-beef-agar (formulation no. B244, Difco Laboratories Inc., Detroit, Mich.). The organisms were subcultured at 4-week intervals, incubated at 28 C for 24 hr, and stored at 5 C thereafter.

Two liquid media were used for the experiments. Yeast-beef broth (Difco formulation no. B244, with the agar omitted) was used in experiments not requiring defined medium conditions. Alternatively, a D-glucose-mineral salts medium of the following composition (in grams per liter) was used: glucose, 1.0; K_2HPO_4, 7.0; KH_2PO_4, 2.0; $MgSO_4 \cdot 7H_2O$, 0.1; $(NH_4)_2SO_4$, 1.0, *p*H 7.2. When required, adjuncts were added aseptically to appropriate volumes of double-strength medium. The media were dispensed in 25-ml portions in 125-ml Erlenmeyer flasks, and these were sterilized by autoclaving at 123 C for 15 min. Incubation was carried out in a New Brunswick reciprocating incubator, operating at 80 oscillations per minute. For particularly critical control of incubation temperature, shaking water baths were used.

Chemicals and reagents. Phloretin and most of the inorganic chemicals used in this study were obtained from British Drug Houses (Canada) Ltd., Toronto. L-Tryptophan, *p*-nitrophenyl-β-glucoside (*p*-NPG), thiamine, riboflavine, nicotinic acid, pantothenic acid, pyridoxine, pteroylglutamic acid, choline, D-biotin, cobal-

amin, mitomycin C, and Merthiolate were supplied by the Sigma Chemical Co., St. Louis, Mo. Ionagar No. 2 was obtained from Oxoid Ltd., London, England, and vitamin-free casein hydrolysate was supplied by the Nutritional Biochemical Co., Cleveland, Ohio. Nondefined medium constituents were obtained from Difco. All other chemicals and reagents were of analytical reagent grade, and the best grade available commercially. They were used as received from the supplier. The water supply was deionized and redistilled in glass.

Screening of strains for the production of white variants. Cell suspensions from 18-hr slopes were prepared in yeast-beef broth to contain 10^4 to 10^5 cells/ml, and 0.1 ml of such suspension was used to inoculate triplicate 125-ml Erlenmeyer flasks containing 10 ml of yeast-beef broth. Each strain studied was grown for 22 hr in parallel series of flasks, incubated, with shaking, at 30 and 37 C, respectively. After incubation, samples were removed from the cultures, and 10-fold serial dilutions were prepared in sterile 0.85% sodium chloride. Samples (0.1 ml) of the dilutions were spread on the surface of yeast-beef-agar plates with an angled glass rod. The plates were incubated at the temperature at which the corresponding liquid cultures had been grown. Examination for the presence of white colonies was undertaken 72 hr later. Although good growth had occurred and color differentiation was normally possible after 24 hr of growth, pigment production was maximal at approximately 72 hr, permitting the unequivocal differentiation of the variant and the parent strain. Colony counts were performed, and the percentage of white variants present was calculated.

Determination of effect of temperature on formation of white variants. To determine the effect of temperature on the formation of white variants, experiments were carried out with *E. herbicola* Y46. The organism was inoculated (as described above) into 20 ml of yeast-beef broth in triplicate 125-ml Erlenmeyer flasks which had previously been equilibrated at their respective experimental temperatures. The inoculated flasks were incubated with shaking for 22 hr. Tenfold serial dilutions were prepared in 0.85% sodium chloride, and samples (0.1 ml) were plated on yeast-beef-agar. After incubation at 30 C for 72 hr, the resulting colonies were counted, and the percentage of white variants was determined.

Cultural and physiological character of E. herbicola Y46 and its white variants. One hundred and seventy-three cultures, including the parent strain, 84 yellow isolates from various experiments in this study, and 88 white isolates produced as a result of growth of *E. herbicola* Y46 at 37 C were subjected to the tests listed in Tables 3 and 4. This survey was performed twice, and those characteristics which varied between isolates were repeated once more (Table 4). Most of the tests are standard and are described fully in the references cited in Table 3. Phloretin hydrolase (5, 6) was detected by treating 0.3 ml of a suspension of approximately 2×10^8 24-hr cells/ml in 0.05 M potassium phosphate buffer (*p*H 6.7) with 0.3 ml of 0.002 M phloretin in 0.001 N NaOH and 0.4 ml of 0.05 M potassium phosphate buffer (*p*H 6.7). After incubation at laboratory temperature for 5 min, the phloroglucinol liberated was detected by adding 2.0 ml of concentrated hydrochloric acid and 0.2 ml of 1.0% vanillin (w/v) in absolute ethanol (6). The production of an orange-brown color indicated a positive reaction. β-Glucosidase was detected in the same cell suspensions by adding 0.9 ml of the suspension to 0.1 ml of 0.01 M *p*-NPG prepared in the same buffer. A positive test was indicated by the formation of a yellow color after 30 min of incubation at laboratory temperature. For all enzyme tests, control experiments, in which a boiled cell suspension was used, were performed for each isolate.

Nutritional requirements of the white variants. It was observed early in this study that the white variants would not grow on the defined medium described above, but would grow well on yeast-beef-agar. To determine the identity of the limiting compound(s), the procedure of Lederberg (23) was used. The basal defined medium was supplemented either with amino acids, in the form of filter-sterilized vitamin-free casein hydrolysate (0.1%) to which was added L-tryptophan to a final concentration of 5.0 μg/ml medium, or with a mixture of growth factors consisting of thiamine (1.0 μg/liter of medium), riboflavine (50 μg), nicotinic acid (10 μg), pathothenic acid (10 μg), pyridoxine (10 μg), pteroylglutamic acid (1.0 μg), choline (200 μg), D-biotin (1.0 μg), and cobalamin (1.0 μg). In later experiments, the growth factors were incorporated into the basal medium singly to identify the required compound specifically. In all experiments, control plates containing the unsupplemented basal medium, and plates containing basal medium with 0.3% (w/v) yeast extract were inoculated with each isolate studied.

Cell suspensions from 24-hr yeast-beef-agar slopes were prepared in sterile phosphate buffer solution of the same concentration as that in the defined medium, to give a turbidity corresponding to 0.5 on the McFarland comparator scale (22). These suspensions were incubated for 4 hr at 30 C and then streaked on the appropriate medium. The plates were observed after 2 and 5 days of incubation at 25 C.

Serological procedures. Immunizing antigens were prepared from 24-hr yeast-beef-agar slopes of the appropriate organisms. All operations in the production of the antigens were performed aseptically. The cells were suspended in 0.85% sodium chloride solution and washed twice by centrifugation. The cells were suspended in physiological saline and diluted to give approximately 2×10^8 cells/ml. This suspension was filtered through lens tissue and dispensed into serum bottles. These preparations were used for intravenous administration to rabbits (two animals for each antigen), and 1.0 ml was administered on each of days 1, 2, 3, 12, 13, 14, 25, 26, and 27. Small test bleedings were made on days 17, 20, and 31, and a large (20 ml) sample taken on day 38 from each animal. The antisera from the duplicate animals receiving each antigen were pooled, and the γ-globulin fraction was separated and concentrated fivefold by the procedure of Goldman (14).

The serological relationships between bacterial strains were investigated by the gel diffusion procedure, by using 1.0% Ionagar No. 2 in 0.85% sodium chloride, containing 0.01% Merthiolate as preservative, essentially by the methods used previously (13).

Isolation of virulent and temperature phages. Virulent phages were isolated from raw sewage by standard procedures (1). Temperate phages were obtained by treatment of cultures in the defined medium (*see above*) with

mitomycin C. The procedure of Otsuji et al. (25) was followed.

RESULTS

Production of white variants by strains of E. herbicola. Of the nine strains of *E. herbicola* tested, six produced white variants when grown in yeast-beef broth at 37 C (Table 1). No variants appeared at 30 C. Some of the variant colonies were sectored in yellow and white, and microscopic examination showed the variants to be small, gram-negative rods, occurring singly or in pairs, very similar to the parent strains. Further experimentation was limited to strain Y46, as this organism has previously been the subject to extensive study (5-7). The numbers assigned to the variant strains obtained from this organism are of no significance, other than for identification purposes.

Effect of temperature on the production of variants of E. herbicola Y46. The results of one representative experiment are summarized in Table 2. White variants were produced only at 34 and 37 C, of the temperatures tested, and the maximum observed frequency of occurrence was 8.8%. In other experiments, this value has been observed as high as 15%. Higher frequencies were also shown by recycling yellow colonies, obtained after one incubation period at 37 C through a second such period. It is apparent that a critical temperature exists between 29 and 34 C: white variants have never been observed at 30 C or below, in spite of extensive experimentation with *E. herbicola* strains in many contexts over a number of years.

Cultural and physiological characteristics of E. herbicola Y46 and its white variants. Those tests which gave the same results for all isolates tested are listed in Table 3, together with the appropriate results. The results of those tests in which

TABLE 1. *Details of the strains of Erwinia herbicola used in this study, and their sensitivity to incubation at 37 C*

Strain no.[a]	Source	White variants[b]
Y13	Pear bud	Yes
Y17	Pear bud	Yes
Y46	Apple canker	Yes
Y57	Pear twig	Yes
Y69	Apple twig	No
Y74	Mountain ash twig	No
Y163	Pear canker	Yes
Y178	Pear twig	Yes
Y185	Pear fruitlets	No

[a] All these strains had been maintained as described in Materials and Methods since their original isolation. They were all isolated from material obtained in Ontario.

[b] Production at 37 C.

TABLE 2. *Effect of temperature of incubation on production of white variants of Erwinia herbicola Y46 grown in yeast-beef broth shake cultures*

Incubation temp (C)	Viable count/ml		White variants (%)
	Yellow	White	
25	7.5×10^9	$<1.0 \times 10^6$[a]	<0.01[a]
29	7.2×10^9	$<1.0 \times 10^6$[a]	<0.01[a]
34	3.0×10^9	1.5×10^8	4.7
37	4.1×10^7	4.0×10^6	8.8
42	7.7×10^2	<10[a]	<0.13[a]

[a] Figures represent lower limit of detection of white variants under conditions of the experiment.

TABLE 3. *Cultural and physiological characters of 172 yellow and white derivatives of Erwinia herbicola Y46*[a]

Test	Reaction	Reference
Gram reaction	Negative	28
Urease production	Negative	8
Malonate utilization	Positive	27
Methyl red test	Negative	28
Voges-Proskauer test	Positive	28
Indole production	Negative	28
Nitrate reduction	Positive	28
Phenylalanine deamination	Positive	27
Kligler's iron agar	Glucose utilized, H_2S-negative	27
Hugh and Leifson's test	Fermentation	27
Starch hydrolysis	Negative	27
Gelatin hydrolysis	Negative	27
Deoxyribonuclease production	Negative	21
Catalase test	Positive	27
Peroxidase	Positive	27
β-Glucosidase	Positive	This paper
Phloretin hydrolase	Positive	This paper

[a] All isolates investigated gave similar results with each of these tests.

the organisms varied, namely, the pigmentation of the isolate, motility, utilization of citrate, tolerance of 7.5% sodium chloride, and the prototrophic or auxotrophic nature of the isolate, are summarized in Table 4. All the individual strains used in this survey are not listed in this paper, but those strains which received special attention are noted, and the phenotypic groups in which they fall in the classification in Table 4 are indicated.

Among those isolates which retain pigmentation after incubation at 37 C (i.e., groups 1 to 4 in Table 4), no discernable pattern or relationship between the characters lost or retained is apparent. The main characteristic of the white variants (groups 5 to 7, Table 4), beside their lack of pigmentation, is their auxotrophic nature. Over 200 white variants were checked for this property, and all failed to grow on the defined minimal medium. In every case the ability to grow on this medium was restored by the incorporation of thiamine. However, in no case did thiamine give rise to pigmentation of these variants. Other

TABLE 4. *Variations in cultural and physiological characters of 172 yellow and white derivatives of Erwinia herbicola Y46 produced by incubation at 37 C*

Group	Phenotype[a]	No. of characters lost	No. of isolates in group	Isolates used in the present study[b]
1	$Y^+M^+C^+S^+P^+$	0	60	Y46 (parent strain), Y46/254, Y46/267, Y46/292, Y46/388
2	$Y^+M^+C^+S^-P^+$	1	1	
3	$Y^+M^+C^-S^+P^+$	1	4	
4	$Y^+M^+C^-S^-P^+$	2	20	Y46/381, Y46/394, Y46/410
5	$Y^-M^+C^+S^+P^-$	2	2	
6	$Y^-M^+C^-S^+P^-$	3	43	Y46/1, Y46/5, Y46/20, Y46/24, Y46/29, Y46/37, Y46/63
7	$Y^-M^-C^-S^+P^-$	4	43	Y46/46, Y46/70, Y46/83, Y46/100, Y46/113, Y46/124

[a] The characters observed are coded as follows: Y, yellow pigmentation on yeast-beef-agar culture; M, motility in motility agar (Difco no. B450); C, utilization of citrate in Simmon's citrate agar (Difco no. B91); S, tolerance of 7.5% sodium chloride in yeast-beef-agar; P, prototrophy. Superscripts (+) or (−) indicate positive or negative results, respectively.

[b] Numbers, assigned to isolates on an arbitrary basis, have no significance other than identification.

characters, designated M, C, and S in Table 4, show a similar degree of variation to that seen among the yellow variants.

Sensitivity of E. herbicola to bacteriophages. A virulent phage, isolated from sewage and designated Y46/C1, gave rise to clear plaques in lawns of *E. herbicola* Y46. The phage was very similar, morphologically, to the T-even phages of *Escherichia coli*, as demonstrated by electron microscopy (the cooperation of W. J. Vail is gratefully acknowledged). All the white variants of *E. herbicola* tested were lysed by this phage, with an essentially similar efficiency of plating (Table 5). These variants were insensitive to the temperate phage Y46/(E1), isolated from *E. herbicola* Y46 after treatment with mitomycin C. The only organism tested which was susceptible to this phage was the indicator strain *E. herbicola* Y178.

Serological characteristics of the variants of E. herbicola Y46. The following isolates were studied, including five random representatives from each of groups 1, 6, and 7 and three from group 4: group 1—Y46 (parent strain), Y46/254, Y46/267, Y46/292, Y46/388; group 6—Y46/5, Y46/24, Y46/29, Y46/37, Y46/63; group 7—Y46/46, Y46/70, Y46/83, Y46/113, Y46/124; group 4—Y46/381, Y46/394. Y46/410 (Table 4).

The results of gel diffusion experiments with antibodies against *E. herbicola* Y46 and the white variant Y46/70 are illustrated in Fig. 1. The experiments involving the white variants Y46/5, Y46/24, and Y46/124 gave essentially similar data. Three main conclusions can be drawn from these experiments. (i) All the variants tested have a close serological relationship with the parent strain, and among themselves. (ii) A few variants, although still closely related to the parent strain, have lost one of their diffusible antigenic components, indicated by the arrows in Fig. 1. [In this category falls the white variant Y46/37 (group 6, Table 4), and the yellow variants Y46/394 and Y46/410 (both in group 4, Table 4).] (iii) The variations observed in the complements of diffusible antigens bear no apparent relationship to variations in other characteristics.

DISCUSSION

E. herbicola Y46 gives rise to white variants at high frequency on incubation at temperatures above those optimal for growth, and the loss of pigmentation is always accompanied by thiamine auxotrophy in these variants. There appears to be no pattern relating the other characters which are subject to variation in this context, namely utilization of citrate, sodium chloride tolerance, motility, and the complement of diffusible antigens.

Genetic changes brought about by incubation at supraoptimal temperatures have been shown for several bacteria, including *Staphylococcus aureus* (9, 10, 24) and *Bacillus subtilis* (29). In the case of *S. aureus*, the genetic alterations involved the elimination of an extrachromosomal episome or plasmid, and the characteristic of most significance which was affected was the loss of the enzyme penicillinase (9, 10, 24). Neither pigmenta-

TABLE 5. *Sensitivity of Erwinia herbicola Y46, Y178, and eight white variants of Y46 to phages Y46/C1 and Y46/(E1)*

Host	Plaque-forming units/ml	
	Phage Y46/C1	Phage Y46/(E1)
Y46	8.0×10^7	$< 1.0 \times 10^4$ [a]
Y46/1[b]	9.0×10^7	$< 1.0 \times 10^4$
Y46/5[b]	13.3×10^7	$< 1.0 \times 10^4$
Y46/20[b]	7.0×10^7	$< 1.0 \times 10^4$
Y46/24[b]	9.0×10^7	$< 1.0 \times 10^4$
Y46/46[c]	11.3×10^7	$< 1.0 \times 10^4$
Y46/70[c]	12.6×10^7	$< 1.0 \times 10^4$
Y46/100[c]	13.3×10^7	$< 1.0 \times 10^4$
Y46/124[c]	8.3×10^7	$< 1.0 \times 10^4$
Y178	6.5×10^7	18.6×10^7

[a] This figure represents the lower limit for the detection of phages by the titration procedure followed.

[b] Selected arbitrarily from group 6, Table 4.

[c] Selected arbitrarily from group 7, Table 4.

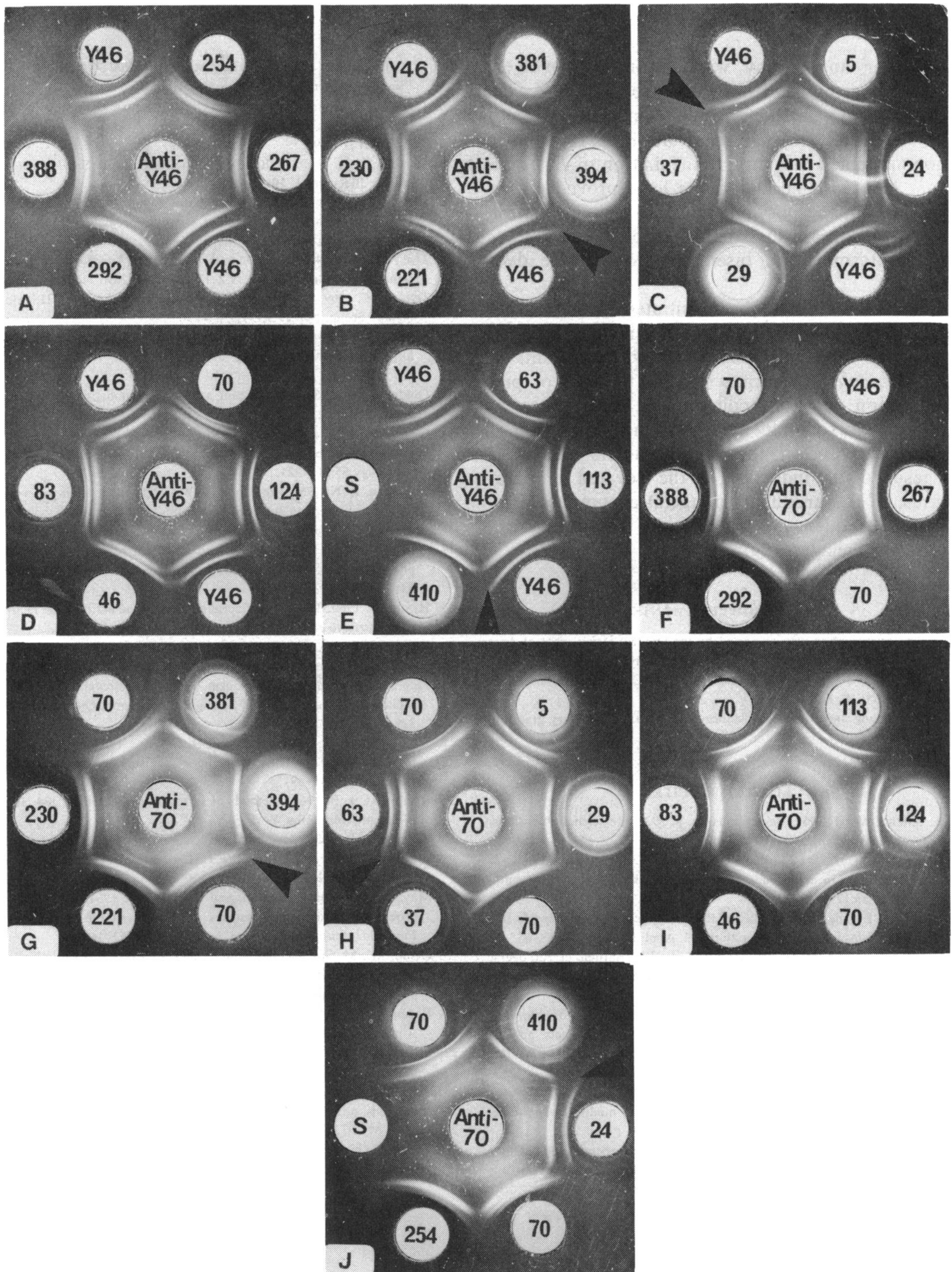

FIG. 1. *Gel diffusion patterns produced by cell suspensions of representative pigmented and white variants of E. herbicola Y46 in 1% agar-0.85% sodium chloride-0.01% Merthiolate medium. The peripheral wells contained thick suspensions of the appropriate strains in saline. Abbreviated culture references are given at each antigen well, e.g., Y46 refers to E. herbicola Y46, and 254 to E. herbicola Y46/254. The center wells contained γ-globulin fractions prepared from rabbit anti-E. herbicola Y46 serum (Anti-Y46) and anti-E. herbicola Y46/70 serum (Anti-70). The plates were incubated at 25 C for 4 days. The isolates here designated 5, 24, 37, 46, 63, 70, 83, 113, and 124 were white, and those designated 254, 267, 292, 381, 388, 394, and 410 were yellow-pigmented derivatives of E. herbicola Y46. Wells containing saline only are indicated by S. The arrows indicate the points where isolates 37, 394, and 410 show their serological distinction from E. herbicola Y46 and Y46/70 (B, C, E, G, H, and J). Isolates 221 and 230 were not derived by procedures described in this paper, and are not relevant to the present investigation.*

tion nor auxotrophy was apparently involved. Acridine dyes have been used to eliminate such extrachromosomal elements from bacterial cells (9, 18, 20, 24, 26). However, attempts to produce nonpigmented variants by incubation of *E. herbicola* Y46 with acridine dyes have proved unsuccessful, but the possibility of plasmid elimination from the organism cannot yet be ruled out, as some plasmids are insensitive to acridine dyes (19, 24).

Experiments have been initiated in these laboratories to test two hypotheses which seek to explain the mechanism by which white variants of *E. herbicola* Y46 arise: (i) that the loss of pigmentation and the concomitant occurrence of thiamine auxotrophy are due to the elimination of plasmid(s), and (ii) that the characters are lost as a result of the induction, at the elevated temperatures, of a transducing phage(s) accompanied by a high frequency of survival of the cells from which the phages originate.

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