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Inheritable instability of colony morphology of *Xanthomonas campestris* pv. *citri*

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Abstract. The typical colony morphology of *Xanthomonas campestris* pv. *citri* was large yellow. Occasionally, however, small white colonies appeared in the midst of the overwhelming majority of large yellow colonies. This change of colony morphology is apparently the result of an unstable mutation that manifests unpredictably in some cell populations. Once arisen, this unstable mutation brings about, in progenies of the originally mutated populations, an alternation of the colony morphology between small white and large yellow at a high frequency for many generations. A similar phenomenon is also found in *Xanthomonas campestris* pv. *citri* XW47 cells carrying the filamentous phage Cf16-v1. The possibility that this unstable mutation affecting the colony morphology may have been generated by a transposable element on a plasmid indigenous to *Xanthomonas campestris* pv. *citri* XW47 is discussed.

Key words: Bacteriophage Cf16; Colony-morphology instability; *Xanthomonas campestris* pv. *citri.*

Introduction

Xanthomonas campestris pv. *citri* XW47, a causative agent of citrus bacterial canker, regularly forms large-yellow colonies on modified LB medium. After a three-day incubation period at 28°C, the colony grew to about 2 mm in diameter. At this stage, occasionally barely visible small white colony appeared spontaneously. The colony morphology as manifested in the progeny of these small white colonies became an unstable characteristic hereafter. It oscillates back and forth between small white and large yellow at a very high frequency in subsequent propagation of individual progeny cells. This alteration of colony morphology is apparently the result of an unstable mutation with a high reversion rates in both directions. As this instability in colony morphology is inherited continuously for at least 500 generations during consecutive of cell transfer.

Instability of colony morphology has also been observed in XW47 cells and phage infected cells (Wu, 1960; Wu *et al.*, 1985 and Dai *et al.*, 1988). We reported that cells recovered from phage carrying sector-colonies may transform into four types of progeny cells each with a different colony morphology (Dai *et al.*, 1988). The progeny colony morphology is correlated with the copy number of phage genome existed in each cell type. The irregular appearance of small white and mosaic sectored colonies indicates that progeny with various colony morphologies can be generated from a single cell.

A transposable element has been identified on the indigenous plasmid of *X. campestris* pv. *citri* XW45 (Tu *et al.*, 1989). The rearrangement of an indigenous plasmid's fragment has been found to occur in response to the phage infection in *X. campestris* pv. *citri* XW47. The interaction of this indigenous plasmid with a recombinant clone carrying a fragment of phage Cf16 has also been shown (Dai and Chiang, 1990). We postu-

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late that a transposable element may be responsible for the instability and the high mutation frequency of colony morphology in XW47. This genetic element may be activated spontaneously and unpredictably during normal cell growth or as the result of phage infection.

Materials and Methods

Bacteria and Lysogens

X. campestris pv. *citri* strain XW47 (Wu *et al.*, 1985) obtained from Dr. W.C. Wu was used in this study. The unstable lysogen LW arisen after infection by phage Cf16 was isolated in our laboratory (Dai *et al.*, 1988).

Media

Tryptone or Luria broth (LB) contained 10 g tryptone, 5 g yeast extract, 5 g NaCl and 1 g glucose in 1 liter water. LB agar plate was prepared with LB containing 1.6% agar.

Colony Morphology and Phage-Producing Capacity

Cells of strain XW47 or lysogen LW were spread or streaked onto LB plate. Small white colonies of XW47 occurred spontaneously after 4 days incubation at 28°C. Stability of colony morphology of it's progenies was determined either by direct of streaking cells onto LB medium or by making a stationary phase cell culture followed by dilution and spreading. Sectoring colony derived from LW lysogen was studied in the same way as colony morphology instability. Cells of single colony was inoculated and grew into a stationary phase for plaque assay as described previously (Dai *et al.*, 1987). Strain XW47 was used as the indicator host.

Determination of Intracellular Phage Genome of Carrier Cell

Total intracellular and phage replicative form (RF) DNAs were isolated as described before (Dai *et al.*, 1987). DNA samples were digested with *Eco*RI restriction endonuclease. The amount of DNA used in agarose gel electrophoresis for the detection of phage genome was 10 μg/slot. Electrophoresis was carried out at 60v for 9 h at room temperature with a 0.8% agarose slab gel (14.5 cm $\times$19.5 cm) prepared in TBE buffer (89 mM tris base, 88 mM boric acid and 2 mM

Table 1. *The random alteration of colony morphology of cells derived from small white colonies*
A small white colony appeared spontaneously among 206 colonies (restreaking 2A). Following the restreaking of this colony, 4.5% of 2465 colonies scored were large yellow, while 95.5% remained as small white (restreaking 3A). Among these small white colonies one was restreaked, 20% of the progeny became large yellow colonies (restreaked 4A). The mutation frequency of colony morphology was studied for 15 consecutive transfers and approximately 50 independent parental colonies were examined. The date of 11 independent experiments were presented here.

Restreaking code	Colony morphology selected along previous transfers										Colony morphology following restreaking		
	1	2	3	4	5	6	7	8	9	10	Number of colony scored	Large yellow (%)	Small white (%)
2A	L										206	99.5	0.5
3A	L	S									2465	4.5	95.5
4A	L	S	S								191	20.0	80.0
5A	L	S	S	S							110	52.0	48.0
5B	L	S	L	S							283	27.0	73.0
6A	L	S	S	S	S						231	48.0	52.0
7A	S	S	S	S	S	S					171	2.0	98.0
7B	L	S	S	S	S	S					539	23.0	77.0
7C	L	S	S	S	L	S					856	92.0	8.0
7D	L	S	S	S	L	S					90	18.0	82.0
11A	L	S	S	S	S	S	S	S	S	S	451	46.0	54.0
11B	L	S	S	S	L	S	S	S	S	S	282	15.0	85.0

L: large yellow colonies; S: small white colonies.

Na_2 EDTA, pH 8). Southern blot analysis with phage RF as a probe was carried out according to the standard procedures (Southern, 1975).

Results

The Variation of Colony Morphology as an Inherited Characteristics

Xanthomonas campestris pv. *citri* strain XW47 normally forms large yellow colonies. This colony morphology persists as a stable character as long as such colonies are selected for continued subculturing. However, small white colonies occasionally appeared in the midst of the normal large yellow colonies (Fig. 1A). The colony morphology of descendants of small white colony was highly variable. The immediate descendants of a small white colony gave rise to a mixture of large yellow and small white colonies (Fig. 1C). The

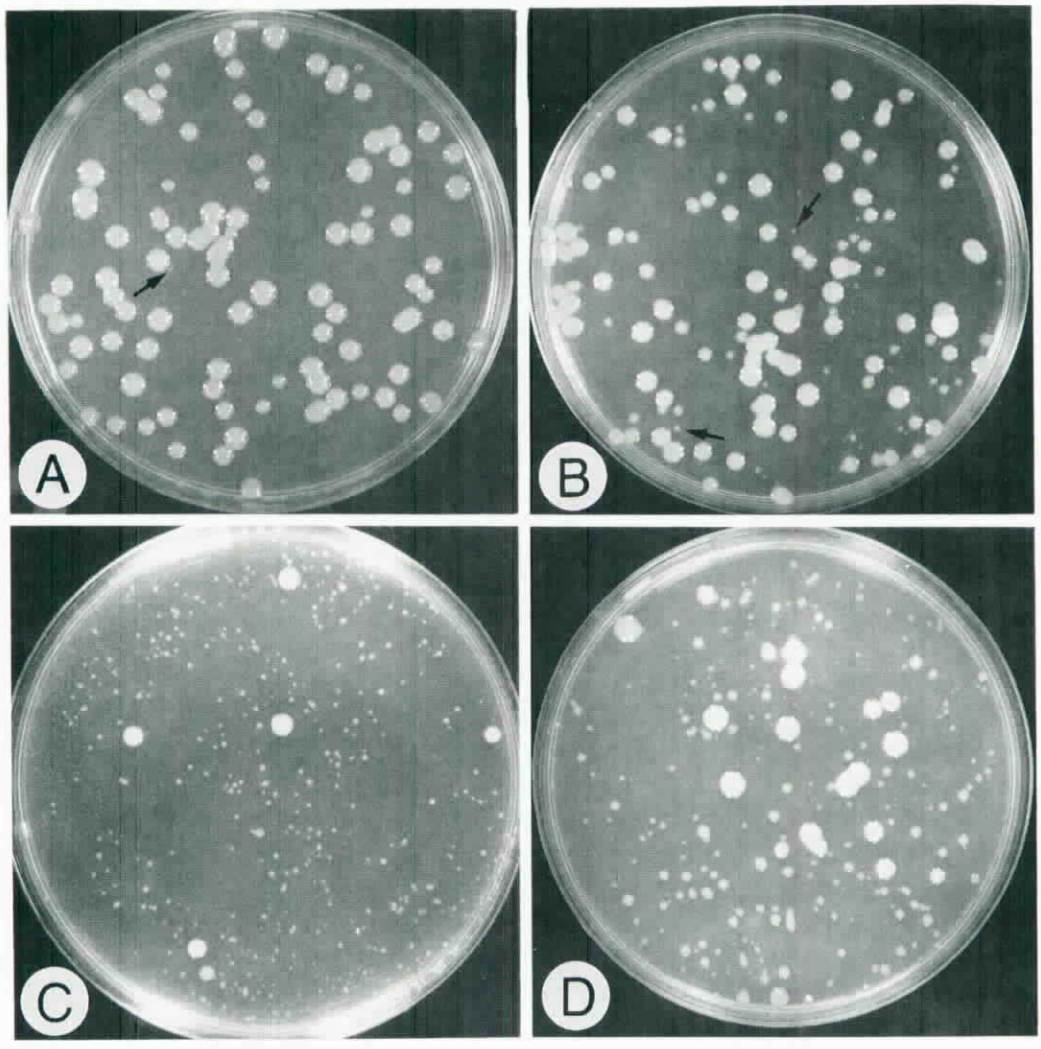

Fig. 1. Spontaneous colony morphology alteration of *X. campestris* pv. *citri*. Wild type XW47 was grown in liquid LB and then a proper number of cells were spread on each plate. A, Five day culture of wild type XW47. Arrow indicates the tiny white colony. B, The progeny of a large yellow colony which was a descendant of a small white colony of plate A. Arrow shows a tiny white colony on a 8 day old plate. C and D, The progeny of 2 independent small white colonies. C and D, represent 5 and 10 day cultures, respectively.

Table 2. *Instability of colony morphology of cells derived from small white colonies*
The stationary-phase liquid cultures of 89 small white and 60 large yellow colonies (all originated from a small white colony) were diluted and plated individually. Between 100 to 1000 progeny colonies were scored after 4-5 days for each colony culture.

Mutation frequency of non-parental type (%)	Number of mutated colony amony total colony examined	Mutation frequency
A. Small white to large yellow		
10-100	29/89	0.32
1-10	34/89	0.38
0.1-1	14/89	0.15
<0.1	12/89	0.14
B. Large yellow to small white		
10-1000	18/60	0.3
1-10	20/60	0.33
0.1-1	12/60	0.2
<0.1	10/60	0.16

Table 3. *Instability of colony morphology of sectored colony segregates*
Parental sectored colonies were obtained from the spontaneous segregation of the LW lysogen (Dai *et al.*, 1988). Each sectored colony was streaked on a plate. After 4-5 day's incubation at 28°C, progeny colonies were scored according to its colony morphology. Total 50 sectored colonies were examined. Data from 10 were presented in this table.

Experiment	Colony morphology of sector-colony progeny (%)				Sample size (colonies)
	Small white	Sectored	Large yellow	Large white	
1	0	100	0	0	175
2	5.1	94.9	0	0	236
3	0	74.3	25.7	0	618
4	0	98.2	1.8	0	109
5	34.0	46.0	8.0	12.0	50
6	34.1	54.6	11.3	0	88
7	0.6	93.5	5.9	0	186
8	0	70.2	23.4	6.4	188
9	1.4	23.6	75.0	0	212
10	7.3	88.7	2.7	1.3	150

cells in these large yellow colonies, however, were still unstable as they continued to segregate out both large yellow and small white colonies (Fig. 1B). Variation of colony size and color remained as an unstable characteristics for at least 15 consecutive transfers, or approximately 500 generations (Table 1). Small white colonies became mucous white after 10 days at 28°C (Fig. 1D).

The possible correlation of a given colony's past history with the frequency of colony-morphology segregation was tested. We examined about 100 individual colonies with various combinations of past history appearing as large yellow and small white colonies. No correlation was found (Table 1). The data present here constituted only a small sample of a much larger body of similar results. It was evident that the high frequency mutational event occurred randomly among different cell populations unrelated to their past history with respect to colony morphology. Furthermore, the ratio of large yellow to small white colonies among the progeny of a given parental cell was a random event. There was no statistic correlation of such ratio among all samples tested. The coefficient of correlation was -0.1823 for the large yellow to small white

colony, whereas the coefficient of correlation was -0.0215 for small white to large yellow colonies resulting from cells originating from small white colony. The mutation frequencies from large yellow colony to small white colony and vice versa among each individual parental colonies examined were shown in Table 2. The mutation frequency varied drastically from colony to colony. Some were as high as 95% while other were less than 0.1%.

The Correlation of Phage Yield and Colony Morphology of Carrier Cell

An unstable Cf16-vl lysogen LW forms sector colonies at a highly variable frequency. The phage titer of these colonies is 1×10^{9} to 2×10^{10} PFU/ml. Cells in LW lysogen derived sector colonies have been found to contain a high copy number of free replicative form (RF) of the phage genome (Dai *et al.*, 1988). The progeny of sector colony derived from LW lysogen gave rise to four different types of colonies: small white (SW), sector (S), large yellow (LY) and large white (LW) with a phage titer of about 2×10^{10}, 1×10^{9} to 2×10^{10}, 1×10^{7} to 1×10^{8}, and 1×10^{4} to 1×10^{6} PFU/ml, respectively. The ratio of each progeny colony type varied from parental colony to colony. Large white colonies occurred only rarely (Table 3).

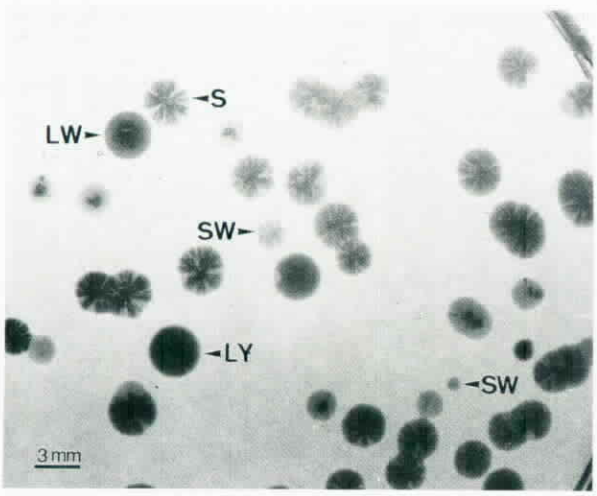

Fig. 2. Colony morphologies of progeny of a sectored colony. An irregular mosaic pattern was shown on sectored (S) and small white (SW) colonies. Large yellow (LY) and the parental unstable lysogen (LW) morphologies were also observed.

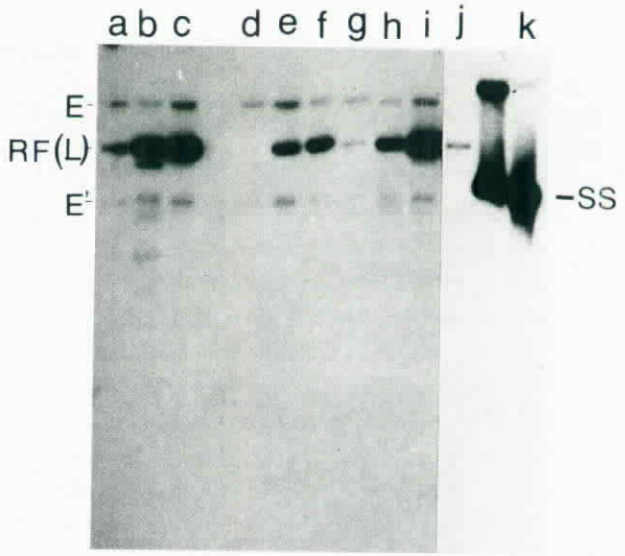

Fig. 3. The presence of phage DNA in progeny of sectored colonies. Total DNA samples were prepared from a small white (lane c) and two sectored colonies, (lanes f and i) and their decedents (lane a, b, d, e, g and h). 10 μg of total DNA and 1 μg of free RF DNA were digested with *Eco*RI. The digested samples were run on a 0.8% agarose slab gel and transferred by blotting onto a nylon membrane followed by hybridization analysis using Cf16 RF as a probe. Lane c is small white colony and lanes b, e, f, h, i, are sectored colonies with phage producing capacitites of about 1×10^{10} PFU/ml. Lanes a and g are large yellow colonies with phage producing capacities of about 1×10^{7} to 1×10^{8} PFU/ml. Lane d is large yellow colony with phage producing capacity 1×10^{5} PFU/ml. E and E': *Eco*RI fragments from the Cf16 carrier cells that hybridize with the RF probe. SS: single stranded phage DNA. RF(L): linear form of phage RF.

Fig. 2 shows the progeny of a sector colony. In some SW colonies, a few small yellow patches were found. Various irregular sector and mosaic patterns appeared in different sector colonies. This means various cell types may segregate out from a single cell. Subcultured cells originated from a white sector of sector colonies often gave rise to more progeny with colony morphologies of SW and S, while cells from a yellow patch often yielded more LY colonies. The number of SW and S type segregates derived from LY type parental cell in sector colonies decreased steadily with consecutive transfers. The phage producing capacity also decreased with consecutive transfers. Eventually the LY type cell became a stable lysogen with a stable

colony morphology (LY) and a phage producing capacity of 1×10^4 to 1×10^6 PFU/ml.

When cell reached lysogenic state, no further colony-morphology variants segregated out from the LY type lysogen. Despite its stability, the LY lysogen was different from another stable lysogen, L7-t5, which releases phage that produces turbid plaque (Dai *et al.*, 1987), whereas the phage released from LY lysogen formed clear plaque. LY lysogen was also different from another lysogen, LW that releases clear-plaque-forming phages but was exceedingly unstable (Dai *et al.*, 1988). Southern blot analysis indicated that cells in the small white and sector colony carried an abundant free RF (Fig. 3). The number of intracellular free RF decreased simultaneously with the graduate change of colony morphology from S or SW to LY. Less and less SW and S colonies segregated out along progressive transfers. Eventually only integrated phage genome was observed when LY type cell reached the lysogenic state. The phage producing capacity is always correlated with copy number of free RF existed in carrier cell.

Discussion

The present study documents the random occurrence and the inheritable instability of colony-morphology alteration in *Xanthomonas campestris* pv. *citri*. It is of interest to note that (i) the frequency of such change, from large yellow colony to tiny small white colony and vice versa, is drastically different from cell to cell even though they were derived from same ancestor (Table 3); and (ii) the mutation affecting the colony morphology occurs randomly and differs drastically among separate cell populations with no regard to their prior history (Table 2). It seems likely that a transposable element may be responsible for this apparently random, unstable and drastic variation of colony morphology. The presence of a tranposable element on a plasmid indigenous to *Xanthomonas campestris* pv. *citri* has been demonstrated in strain XW45 (Tu *et al.*, 1989) and implicated in strain XW47 (Dai and Chiang, 1990).

An apparently correlation between the copy number of phage genome in infected cells and their colony morphology is also demonstrated in the present study. When a high copy number of phage RF is present in the carrier cell, the colony morphology is either SW or S. Irregular mosaic was also shown on both SW and S colonies. Among sector colonies shown in Fig. 2, each individual colony is a vivid record of the segregational characteristic of an individual parental cell. Sector and mosaic colonies with various distinctive pattern have been obtained after merely some twenty generations (from a single cell to a colony of 2 mm in diameter). This result suggests that various high frequency mutational events have occurred in different portion of a colony. Each event represents a uniquely different over-all mutational history that occurred in the twenty generations prior to the emergence of the colony in it's final characteristics. This is very similar to the unstable transposable element found in corn on which various phenotypic patterns were exhibited on the kernel (McClintock, 1956).

Since the colony morphology is correlated with copy number of intracellular RF, we believe that during cell division phage RF might segregate unevenly into the progeny cells. The resulting difference in RF copy number in different cells may then affect the frequency of transposition event. Sector or mosaic colony formed randomly as the result of this unpredicatable distribution of RF among the progeny. It has been demonstrated that the host chromosome possesses only one site for phage RF integration (Dai *et al.*, 1987 & 1988), the variation of colony morphology might be caused by some other unstable secondary factor (s) that is activated by phage infection. Wu has also found the occurrence of for an unstable, high frequency mutation of histidine auxotroph after phage infection (Wu *et al.*, 1985).

We hypothesize that a yellow-pigment producing and a cell-division rate gene in XW47 are interrupted by a transposable element which is activated either spontaneously or through phage infection. The higher the copy number of the phage genome in the carrier cell, the higher the chance for these genes to be interrupted by the activated transposable element. A gradual, continuous elimination of the free RF in the carrier cell reduces the chance for the interruption of these genes by transposable element. This perpetual process leads eventually to the complete elimination of free RF in the carrier cell and the coincident restoration of the previously interrupted genes effecting the pigment production and cell-division-rate regulation in the resulting stable lysogen.

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Literature Cited

Civerolo, E. L. 1985. Indigenous plasmids in *Xanthomonas campestris* pv. *citri*. Phytopathology **75:** 524-528.

Dai, H. and K.-S. Chiang. 1990. Sequence alternation of indigenous plasmid in *Xanthomonas campestris* pv. *citri* in response to filamentous phage infection. Microbios **63:** 21-28.

Dai, H., S. Huang, and K.-S. Chiang. 1988. The characterization of a spontaneously segregating Cf16-vl lysogen in *Xanthomonas campestris* pv. *citri*. Intervirology **29:** 207-216.

Dai, H., S.-H. Tsay, T.-T. Kuo, Y.-H. Lin, and W.-C. Wu. 1987. Neolysogenization of *Xanthomonas campestris* pv. *citri* infected with filamentous phage Cf16. Virology **156:** 313-320.

McClintock, B. 1956. Controlling element and the gene. Cold Spring Harbor Symp. Quant. Biol. **21:** 197-216.

Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:** 503-517.

Tu, J., H.-R. Wu, Y.-C. Chang, R. Lurz, B. Dobrinski, and W.-C. Wu. 1989. Transposable elements of *Xanthomonas campestris* pv. *citri* originating from indigenous plasmid. Mol. Gen. Genet. **217:** 505-510.

Wu, W. C. 1960. Studies on the colonial variants of *Xanthomonas citri* (Hasse) Dowson. Plant Prot. Bull. ROC **2:** 127-136.

Wu, W.-C., Y.-K. Hong, C.-M. Huang, Y.-Y. Ten, T. Lee, S.-F. Chang, C.-H. Wang, and M.-H. Huang. 1985. Phage induced mutations in *Xanthomonas campestris* pv. *citri*. Plant Prot. Bull. ROC **27:** 19-33.

柑橘潰瘍病病原細菌菌落之不穩定性

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柑橘潰瘍病病原細菌在 LB 洋菜培養基上呈現大而黃之菌落。偶爾因自然突變，出現一十分小而白色之菌落。當這突變在菌體中發生後，其子代即不斷以不規則之方式發生菌落變異。相同的情形亦發生在被線狀噬菌體 Cf16-v1 感染之細菌上。我們相信此細菌帶有一跳動子，它是直接或間接造成細胞分裂速度和菌落顏色突變之因素。