Occurrence of a Bacterial Watermelon Fruit Blotch in Florida

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ABSTRACT

Somodi, G. C., Jones, J. B., Hopkins, D. L., Stall, R. E., Kucharek, T. A., Hodge, N. C., and Watterson, J. C. 1991. Occurrence of a bacterial watermelon fruit blotch in Florida. Plant Dis. 75:1053-1056.

Mature watermelon fruit with large, firm, dark green, water-soaked lesions were observed in spring 1989. Losses of up to 50% in marketable fruit occurred in some fields in Florida. A gram-negative, aerobic, rod-shaped, nonfluorescent, oxidase-positive, arginine dihydrolase-negative bacterium was isolated from diseased tissues. In pathogenicity tests, the bacterium produced symptoms in foliage and fruit. Morphological, physiological, and biochemical properties of the bacterium related it to a previously described pathogen of watermelon, *Pseudomonas pseudoalcaligenes* subsp. *citrulli*. However, our strains from watermelon produced a hypersensitive response in tobacco and tomato, whereas the type strain of *P. p. citrulli* did not.

In spring 1989, mature watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) fruit with large, firm, dark green, water-soaked lesions with irregular margins were observed in Florida. Fruit lesions started as small water-soaked areas a few millimeters in diameter which did not extend into the flesh of the melon. Large dark brown areas occurred in portions of the water-soaked tissue on older lesions, which were located on the upper portion of the fruit. As the lesions aged, the periderm often cracked, a white bacterial ooze exuded, and decay occurred in some fruit. Up to 50% of marketable fruit were destroyed in some Florida fields and, as the 1989 season progressed, the disease was reported in southeastern, mid-Atlantic, and mid-western states.

In 1965, Webb and Goth isolated an unidentified bacterium from naturally diseased watermelon seedlings that caused water-soaked spots on foliage (24). In 1967 and 1968, several watermelon fruit with symptoms similar to those seen in Florida in 1989 were reported at Leesburg, FL (2). At that time, a fluorescent bacterium was isolated, but its relationship to the disease was not determined. Schaad et al (14) described a bacterium, *Pseudomonas pseudoalcaligenes* subsp. *citrulli*, isolated from water-soaked lesions in cotyledons of several watermelon seedlings in Georgia and noted a similarity to the pathogen described by Webb and Goth (24). Sowell and Schaad did not observe any lesion development in fruit of plants infected with this bacterium (17). A severe epidemic of a watermelon fruit blotch was reported in 1988 (21,22) on Guam and Tinian, and the causal organism was identified as *P. p. citrulli* (23). The disease was also reported in Australia in 1986 but has not recurred (A. Diatloff, *personal communication*).

The objectives of this study were to determine the cause of watermelon fruit blotch in Florida and to characterize the causal agent through determination of morphological, physiological, and biochemical properties.

MATERIALS AND METHODS

Bacterial strains. Strains were isolated from lesions in watermelon fruit from several locations. Several strains were obtained from A. L. O'Leary (Ohio), E. M. Dutky (Maryland), J. A. Bartz (Florida), R. A. Haygood (South Carolina), and R. X. Latin (Indiana). The type strain of *P. p. citrulli*, watermelon strains, and nonfluorescent pseudomonads used in these studies are listed in Table 1.

Isolation and cultural characteristics. Rind tissue from the advancing margin of lesions in mature fruit was surface-disinfested by rubbing the fruit surface with a paper towel moistened with 70% ethanol. Excised tissue was then triturated in a drop of sterile deionized water. After 15 min, a loopful of the suspension was streaked onto nutrient yeast-dextrose agar (NYDA) (6) and King's medium B agar (KMB) (7). Plates were incubated at 28 C for 2–3 days. Single colonies were transferred and strains were stored in 15% glycerol at −70 C. The growth of the strains was evaluated on nutrient agar (NA), NYDA, KMB, and nutrient glucose agar (NGA).

Foliar pathogenicity test. The pathogenicity of several strains from watermelon and the type strain of *P. p. citrulli* was tested individually on seedlings of Charleston Gray and Royal Jubilee at the two-leaf stage. Bacterial strains were grown for 48 hr on NYDA plates at 28 C. Bacterial cells were suspended in sterile 0.01 M $MgSO_4 \cdot 7H_2O$ adjusted to approximately 10^8 cfu/ml, based on spectrophotometer readings, and misted onto plants until runoff. Control plants were sprayed with sterile 0.01 M $MgSO_4 \cdot 7H_2O$. Plants were covered with polyethylene bags and sealed to maintain high humidity and incubated at 28 C in a growth chamber. After 36 hr, the bags were removed and the plants were placed in a greenhouse to observe symptom development. In a second test, the procedure was similar, with the exception of a cooler incubation temperature (26 C), a lower inoculum concentration (approximately 10^6 cfu/ml), and younger plants (first true leaves emerged).

Cultivar susceptibility. Thirty-six watermelon cultivars were planted in AC 6/10 handiflats (Ball Seed Co., West Chicago, IL) containing 3:1 Jiffy mix/sterilized sand. Three replicates of six plants each per cultivar were grown in a greenhouse at 26 C. After seedling emergence, 20-20-20 soluble fertilizer was applied at recommended rates every 7 days. Plants were inoculated at the third fully expanded leaf stage, approximately 3 wk after sowing. Inoculum from strain H-1 was made by washing bacteria from petri dishes with a 0.85% $CaCl_2$ solution and adjusting to approximately 10^3 cfu/ml. Plants were sprayed until water-soaked at 7.32 kg/cm^2 with a Badger 200 artist airbrush and allowed to dry before returning to greenhouse benches. Data were collected 14 days after inoculation, with ratings based on disease development ranging from 1 to 5 where 1 = restricted necrotic spots <0.05 mm with no chlorosis and 5 = large spreading lesions >15 mm with extensive chlorosis. The experiment was repeated once.

Detached fruit inoculation. Twelve mature asymptomatic Prince Charles watermelon fruit, collected from a field with symptoms of fruit blotch, were used

Florida Agricultural Experiment Station Journal Series R-00860.

Accepted for publication 2 April 1991.

for greenhouse inoculations. Each fruit was marked into four sections perpendicular to fruit length. Twelve treatments were evaluated randomly with four replications. Four uninoculated control treatments consisted of placement of sterile water on four surfaces—unbruised fruit surface, xylene-rubbed fruit surface, lightly sandpapered fruit surface, and below the fruit surface by injection with a 5-cc syringe. The eight inoculated treatments consisted of adding approximately 10^6 cfu/ml of either of two isolates to each treatment type as that of the uninoculated controls. The areas treated with xylene or sandpaper did not exceed a diameter of 2 cm. Injections below the fruit surface were made no deeper than 1 cm with a 5-cc syringe.

After treatment, all fruit were wrapped with wet paper towels, placed individually into plastic bags to retain moisture, and placed on a greenhouse bench where temperatures varied from 15 to 36 C. Results were recorded as symptoms developed. Tissue from the margins of blotches resulting from inoculations were macerated, plated on NA, and the bacterium was grown in pure culture and subjected to several physiological and biochemical tests, including hypersensitivity (8), fatty acid analysis by the Microbial Identification System (MIDI, Newark, DE), pitting in CVP (3), oxidase reaction (6), arginine dihydrolase activity (20), and fluorescence on KMB (7).

Field inoculation experiment. Plants of four watermelon cultivars, Charleston Gray, Mickylee, Prince Charles, and Royal Jubilee, were inoculated in the fall in the field at 5 wk of age. Inoculum prepared from two pathogenic strains, adjusted to approximately 5×10^5 cfu/ml, was applied to foliage with a backpack sprayer. Individual fruit of each cultivar (approximately 10 fruit per genotype) were inoculated 2 wk after pollination by gently spraying the fruit surface until runoff with a hand-held pump sprayer. Inoculations were performed between 5 and 6 p.m. Fruit remained on the vines in the field and were observed for symptoms, with final evaluations made 3 wk after inoculation.

Table 1. Bacterial strains tested in watermelon fruit blotch studies

Bacterium	Strain	State
Watermelon pathogen	8175-2	FL
Watermelon pathogen	8185-2	FL
Watermelon pathogen	8191	FL
Watermelon pathogen	3589	FL
Watermelon pathogen	3598	FL
Watermelon pathogen	3603	FL
Watermelon pathogen	3708	FL
Watermelon pathogen	WFB89-1	FL
Watermelon pathogen	1	FL
Watermelon pathogen	1752	FL
Watermelon pathogen	WF1	IN
Watermelon pathogen	WF2	IN
Watermelon pathogen	3861	MD
Watermelon pathogen	3862	MD
Watermelon pathogen	8193	SC
Watermelon pathogen	WM-1	SC
Watermelon pathogen	1438-48	SC
Watermelon pathogen	H-1	SC
Pseudomonas acidovorans	ATCC 15668	
P. cepacia	ATCC 25416	
P. cepacia	ATCC 17759	
P. cepacia	ATCC 10856	
P. gladioli	ATCC 19302	
P. pseudoalcaligenes	ATCC 17440	
P. pseudoalcaligenes subsp. *citrulli*	ATCC 29625	

Morphological, physiological, and biochemical properties. The following tests were conducted on all or a representative selection of individual strains: gram reaction (19), fluorescence on KMB (7), flagella stain (15), pitting in CVP (3), arginine dihydrolase activity (20), oxidase reaction (6), levan production (10), gelatin hydrolysis (12), oxygen requirement (12), lipolytic activity (16), maximum growth temperature (13), starch hydrolysis (12), nitrate reduction (11), hypersensitivity on tobacco and tomato (8), and use of individual carbon sources (11).

Fatty acid determination. Each strain was streaked onto nutrient agar and a single colony was transferred onto trypticase soy broth agar. After 24 hr of incubation at 28 C, approximately 40 mg of cells (wet weight) were transferred to a 13×100 mm glass tube fitted with a Teflon-lined screw cap. Methyl esters were then derived from the bacterial fatty acids in a four-step extraction protocol according to the instructions accompanying the Microbial Identification System.

Bacterial fatty acid methyl esters were separated by a Hewlett Packard 5890 GLC fitted with a capillary column (Ultra 2, cross-linked 5% phenyl methyl silicone, 25 M $\times$ 0.2 mm i.d. $\times$ 0.33-μm film thickness) and a flame ionization detector. Temperature programming began at 170 C and increased by 5 C per min to a final temperature of 270 C for 2 min. The hydrogen carrier gas flow rate was 20 ml/min. Sample volume of 2 μl of the extract was automatically injected with split ratio 100:1.

RESULTS AND DISCUSSION

Strains from watermelon caused foliar symptoms of similar severity in growth chamber/greenhouse inoculation tests. Symptoms on both cotyledons and true leaves were similar to those described previously (14). Water-soaked lesions became necrotic with symptoms slightly more severe on Charleston Gray than on Royal Jubilee. Control plants had no symptoms. In the first experiment, the type strain of *P. p. citrulli* produced no symptoms on foliage, whereas in the second experiment, mild symptoms were observed.

When 36 watermelon genotypes were inoculated to evaluate seedling foliar susceptibility, lesions developed on all cultivars. Variation between experiments prevented designation of resistant genotypes. However, in a replicated greenhouse test with *P. p. citrulli*, Sowell and Schaad (17) reported that two USDA Plant Introduction (PI) accessions were resistant, and the cultivar Congo reacted similarly to the PIs. Congo and the two PIs were more resistant than Charleston Gray or Jubilee.

Symptom development occurred in detached, mature watermelon fruit when fruit were wounded before inoculation. Control treatments, including placement of sterile water on healthy fruit, xylene-rubbed fruit, or lightly sandpapered fruit, and water injected below the fruit surface produced no symptoms. In addition, water suspensions of strains 1 and 1752 placed on healthy, xylene-wounded, or nonwounded mature fruit produced no symptoms. Strains 1 and 1752 applied to sandpapered fruit surfaces induced water-soaked blotches after 2 days and produced lesions 2.5 and 3.1 cm in diameter, respectively, 14 days after inoculation. Symptoms were more severe when the bacterium was injected into the rind. Strains 1 and 1752, when injected into the rind, produced water-soaked blotches after 2 days and lesions 8.9 and 9.4 cm in diameter, respectively, 14 days after inoculation. Three weeks after inoculation, fruit injected with either isolate were hollow and rotted. Bacteria isolated from the margins of blotches reacted similarly to strains 1 and 1752 in physiological and morphological tests. Wall and Santos (21,22) also reported that symptoms developed only on watermelon fruit wounded with a dissecting needle or by scraping. Sowell and Schaad (17) reported that *P. p. citrulli* produced a brown, firm, interior necrosis when injected into the interior of mature, harvested watermelon fruit.

Artificial wounding was not necessary for disease development on immature melons in field inoculations. Water-soaked spots first appeared 72 hr after the inoculum was sprayed on the fruit surface. Approximately 50% of fruit of each genotype developed symptoms. The number of water-soaked spots varied from one or two spots to hundreds of spots per fruit, regardless of genotype. Some lesions encompassed the entire melon surface except for the area contacting the soil. Of the inoculated melons that did not develop typical fruit blotch, most had no visible symptoms, but a few had very small (<3 mm) spots that did not develop further. *P. p. citrulli* caused severe symptoms on watermelon only in the seedling state in the field (17).

Results of investigations on morphological, physiological, and biochemical properties of the watermelon strains compared with type strains are shown in Table 2. Strains of the pathogenic bacterium from watermelon rind tissue

grew on common media (NA, NYDA, KMB, and NGA) were rod-shaped, obligately aerobic, and did not fluoresce on KMB. They were arginine dihydrolase negative, oxidase positive, gram-negative, caused no pitting on CVP, did not reduce nitrate, did not produce levan, grew at a maximum of 39 or 42 C, and produced white colonies on NGA. All watermelon strains tested were lipolytic, starch hydrolysis was negative, and gelatin hydrolysis was very slight. One polar flagellum was present. A hypersensitive response was produced in tobacco and tomato by all watermelon strains in 24 hr but not by the type strain of *P. p. citrulli*.

Carbon source usage was evaluated for 15 strains collected in the spring of 1989 from Florida and southeastern, mid-Atlantic, and midwestern states, and the type cultures of *P. p. citrulli* and *P. pseudoalcaligenes*. Bacteria known to be positive for most carbon sources tested were included as controls. The *P. p. citrulli* type strain and the watermelon strains showed a broader use of carbon sources in our tests than was characteristic of *P. pseudoalcaligenes*. The similarity indices, based on 50 sole carbon sources, for the 15 watermelon strains were as follows: 76% similar to *P. p. citrulli*, 54% similar to *P. pseudoalcaligenes*, 42% similar to *P. cepacia* (Burkholder) Palleroni and Holmes, and 40% similar to *P. gladioli* Severini. Florida watermelon strains were 92, 90, and 90% similar to South Carolina, Indiana, and Maryland strains, respectively. Similarity of Florida strains to *P. p. citrulli* was 80%, whereas South Carolina, Indiana, and Maryland strains were 81% similar to *P. p. citrulli*.

The fatty acid profiles of 24 strains of the watermelon pathogen matched consistently to the *Chromobacterium violaceum* Bergonzini entry in the MIDI reference library, although similarity indices were low (<0.3). *P. acidovorans* den Dooren de Jong was a distant second choice (<0.2), and neither *P. alcaligenes* Monias nor *P. pseudoalcaligenes* were listed as possible matches by the MIDI computer. The profiles of the watermelon pathogen had a mean similarity index of 0.591 to *P. p. citrulli*, the library of which was composed of repeated extractions of the type strain.

The mean percentages of predominant fatty acids of the watermelon pathogen and comparative bacteria are summarized in Table 3. Fatty acid profiles of the watermelon bacterium were quantitatively similar to those of *C. violaceum*, *P. acidovorans*, and *P. p. citrulli*. The lower levels of 16:1 *cis* 9 and 16:0 and higher levels of Summed Feature 7 (a monounsaturated 18 carbon fatty acid having several *cis-trans* isomers) in cells of *P. alcaligenes* and *P. pseudoalcaligenes* quantitatively segregate them from the other bacteria. The amount of 15:0 and Summed Feature 7 fatty acids are lower and higher, respectively, in the watermelon strains than in *P. p. citrulli*.

For all samples listed in Table 3, approximately 80% of the total fatty acid profile is composed of three acids: 16:1 *cis* 9, 16:0, and Summed Feature 7. By plotting the mean percent values of these three acids on a three-dimensional graph, the spatial relationships among the bacteria were portrayed (Fig. 1).

Bacterial strains from diseased mature watermelon fruit produced symptoms on inoculated foliage, detached mature fruit, and on immature fruit in the field. Descriptions of symptoms and the causal agent described here agree with those of watermelon blotch reported by other researchers (21–23) to be caused by *P. p. citrulli*. Morphological, physiological, and biochemical properties of the watermelon bacterium are similar to *P. p. citrulli* described by Schaad et al (14) but do differ. Currently, it is not clear at what level these differences should be recognized. Significant differences are that our strains, as well as strains from Guam and Australia, infect mature fruit (G. C. Wall and A. Diatloff, *personal communication*) and cause HR on tobacco and tomato (G. C. Somodi, *unpublished*). *P. p. citrulli* did not appear to incite fruit blotch in the field (17) or cause HR on tobacco (14) and tomato (G. C. Somodi, *unpublished*).

Originally, *P. p. citrulli* was assigned its position based on limited nutritional use of sugars, which is characteristic of the *P. alcaligenes* group (14). Stanier et al (18) found that the *P. acidovorans* group has characteristics similiar to the *P. alcaligenes* group based on limited usage of sugars as sole carbon sources and other characteristics. They concluded that more refined tests were

Table 2. Characteristics of bacterial isolates from watermelon fruit blotch compared with type strains of *Pseudomonas pseudoalcaligenes* and *P. p.* subsp. *citrulli*

Characteristic[a]	Strains tested (no.)	Watermelon strains	Type strain[b]	
			PPC	PP
Fluorescence on KMB	15	–[c]	–	–
Oxidase	15	+	+	+
CVP pitting	15	–	–	–
Gelatin liquefaction	4	S	S	NT
Nitrate reduction	5	–	–	–
Gram reaction	5	–	–	–
Oxygen relationship (aerobic)	4	+	NT	NT
Lipolytic activity	15	+	+	–
Starch hydrolysis	15	–	–	–
Maximum growth temperature	15	8 at 39 C 7 at 42 C	39 C	39 C
Hypersensitivity, tomato	5	+	–	NT
Hypersensitivity, tobacco	5	+	–	NT
Arginine dihydrolase	4	–	–	NT

[a] Where appropriate, positive or negative controls were tested to ensure accuracy of the tests.
[b] PPC = *Pseudomonas pseudoalcaligenes* subsp. *citrulli*, ATCC 29625. PP = *P. pseudoalcaligenes*, ATCC 17440.
[c] – = Negative, + = positive, S = slight, and NT = not tested.

Table 3. Mean percentage of major fatty acids of the watermelon pathogen and comparative strains

	Watermelon strains[a]		CV[b]	*Pseudomonas* strains[c]							
				PPC[d]		PAc[e]		PA[f]		PP[g]	
Fatty acid	Mean	SD	Mean	Mean	SD	Mean	SD	Mean	SD	Mean	SD
10:0 30H	3.98	0.57	2.50	4.09	0.70	3.93	0.49	3.12	0.48	3.50	0.25
12:0	2.55	0.18	3.75	2.74	0.26	2.55	0.23	7.78	0.25	7.80	0.28
12:0 30H	0.01	0.05	2.39	...	...	...	...	3.61	0.13	4.09	0.48
14:0	1.61	0.15	2.70	2.32	0.24	2.20	0.50	0.81	0.03	0.07	0.15
15:0	3.24	0.63	0.96	6.13	0.64	0.43	0.67	...	...	0.61	0.13
16:1 *cis* 9	42.01	0.58	39.54	41.73	0.64	43.20	0.59	25.64	0.90	23.36	2.17
16:0	34.57	0.51	29.08	33.10	1.50	32.54	1.89	16.09	0.15	12.49	1.08
17:0 *cyclo*	0.58	0.48	1.52	...	...	...	...	...	...	...	...
Summed Feature 7[h]	9.38	0.08	15.12	6.97	0.75	15.05	1.11	41.55	0.48	44.32	1.81

[a] Twenty-four strains were analyzed once.
[b] CV = *Chromobacterium violaceum*. Values are the mean of the MIDI reference library entry.
[c] PPC = *Pseudomonas pseudoalcaligenes* subsp. *citrulli*, ATCC 29625; PAc = *P. acidovorans*, ATCC 15668; PA = *P. alcaligenes*; and PP = *P. pseudoalcaligenes*, ATCC 17440.
[d] The same strain was analyzed three times.
[e] The same strain was analyzed 11 times.
[f] Values are the mean of the Stall reference library entry.
[g] The same strain was analyzed four times.
[h] A monounsaturated 18-carbon fatty acid having several *cis-trans* isomers.

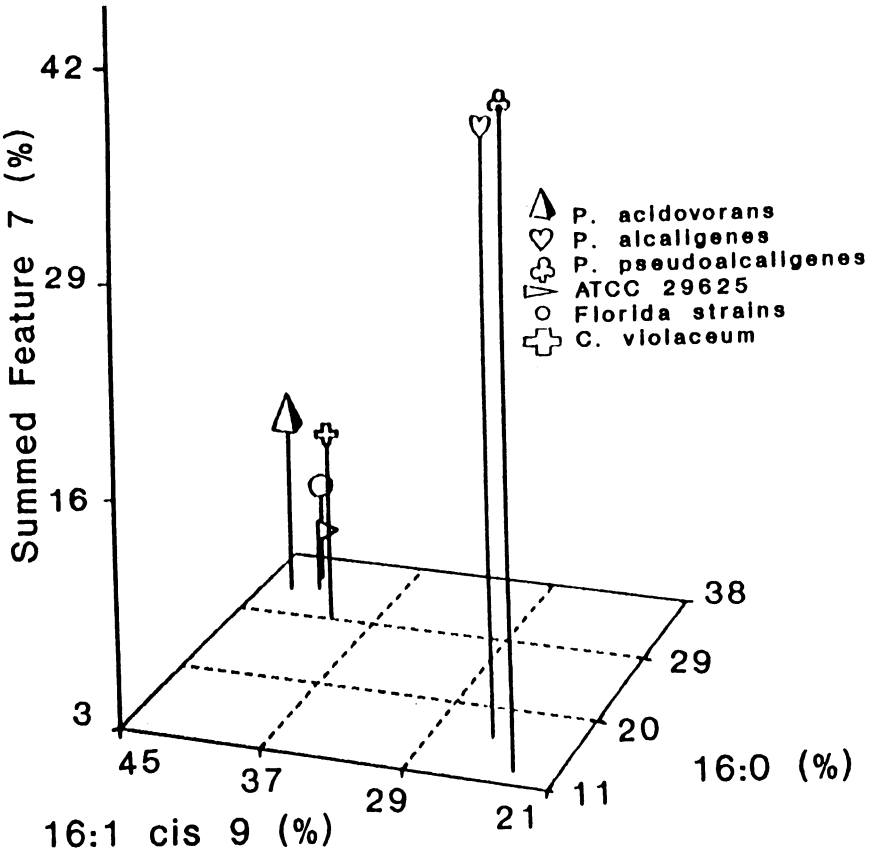

Fig. 1. Comparison of three major fatty acids from the watermelon bacterium (Florida strains) and other related nonfluorescent bacteria.

needed to differentiate these groups. In our study, the similarity of our watermelon strains to *P. pseudoalcaligenes* (52%), *P. alcaligenes* (54%), and *P. acidovorans* (50%) based on carbon usage results in Bergey's Manual of Determinative Bacteriology (1) and Systematic Bacteriology (9) were comparable, supporting the conclusion of Stanier et al (18) that more precise tests are needed.

Fatty acid analyses separated the watermelon organism from *P. pseudoalcaligenes* and *P. alcaligenes*, placing it more closely with *C. violaceum* and, to a lesser extent, with *P. acidovorans*. DeVos et al (5) placed *P. p. citrulli* in the *P. acidovorans* group based on rRNA-DNA hybridizations. In a separate study, DeVos and DeLey (4) also placed *P. acidovorans* and *C. violaceum* in an rRNA superfamily. Thus, fatty acid analyses and rRNA-DNA studies (4) tend to support the close relationship of the watermelon bacterium and *P. p. citrulli*, respectively, with the *P. acidovorans* group.

Correct taxonomic placement of the watermelon fruit blotch bacterium into a species does not appear to be possible at this time based solely on conventional physiological and biochemical tests. More precise characterization of *P. p. citrulli* to determine the most suitable classification will be based on DNA:DNA hybridizations, API 150, and PAGE of whole cell proteins (P. DeVos, *personal communication*).

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