Molecules Involved in the Modulation of Rapid Cell Death in *Xanthomonas*

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In earlier studies from this laboratory, *Xanthomonas campestris* pv. *glycines* was found to exhibit a nutrition stress-related postexponential rapid cell death (RCD). The RCD was exhibited in protein-rich media but not in starch or other minimal media. This RCD in *X. campestris* pv. *glycines* was found to display features similar to those of the programmed cell death (PCD) of eukaryotes. Results of the present study showed that the observed RCD in this organism is both positively and negatively regulated by small molecules. The amino acids glycine and L-alanine as well as the D isomers of valine, methionine, and threonine were found to induce the synthesis of an active caspase-3-like protein that was associated with the onset of RCD. Addition of pyruvate and citrate to the culture medium induced both the synthesis of active caspase-3-like protein and RCD. Higher levels of intracellular accumulation of pyruvate and citrate were also observed under conditions favoring RCD. On the other hand, dextrin and maltose, the hydrolytic products of starch, inhibited the synthesis of the caspase-3-like protein. Addition of glucose and cyclic AMP (cAMP) to the RCD-favoring medium prevented RCD. Glucose, cAMP, caffeine (a known inhibitor of a phosphodiesterase that breaks down cAMP), and forskolin (from the herb *Coleus forskohlii*, known to activate the enzyme adenylate cyclase that forms cAMP) inhibited the caspase enzyme activity in vivo and consequently the RCD process. The addition of glucose and other inhibitors of RCD enhanced intracellular cAMP accumulation. This is the first report demonstrating the involvement of small molecules in the regulation of nutrition stress-related stationary-phase rapid cell death in *X. campestris* pv. *glycines*, which is programmed.

In eukaryotes, programmed cell death (PCD) is a genetically regulated self destruction process for the elimination of damaged or unwanted cells. It plays an important role in the development and maintenance of the integrity of organisms (26, 47, 48). Cells undergoing PCD exhibit a number of biochemical, physiological, and morphological features (19, 32). PCD in a cell is induced by a certain signal(s). The end point of the signaling activity is the induction and activation of caspases (cysteiny aspartate-specific proteases), the proteases that finally execute PCD (8, 30).

Several investigators have reported the occurrence of PCD in bacteria regulated by chromosomal and extrachromosomal toxin-antitoxin pairs of molecules (9, 15, 18, 20, 35, 36, 45, 49). In *Escherichia coli*, such chromosomal toxin-antitoxin systems include *mazEF* (9, 24, 27), *chpBIK* (24), *relBE* (16), *yefM-yoeB* (5, 6, 17), and *dinJ-yafQ* (18). In earlier studies from this laboratory, *Xanthomonas campestris* pv. *glycines*, a plant pathogen, and the etiological agent of bacterial pustule disease of soybean (*Glycine max*), was found to exhibit a nutritional stress-related postexponential rapid cell death (RCD). The RCD in *X. campestris* pv. *glycines* was found to display features similar to those of the programmed cell death (PCD) of eukaryotes (3, 10–14, 29, 33, 39–41). The present study was undertaken to search for molecules that may be involved in the signaling and induction process of the observed rapid cell death (RCD) in *X. campestris* pv. *glycines*. RCD in this organism was found to be positively and negatively regulated by a

number of small molecules. Pyruvate or pyruvate-generating amino acids and citrate induced RCD following synthesis of caspase-3-like protein and the appearance of caspase enzyme activity. Glucose, caffeine, and forskolin inhibited RCD. The inhibitors of RCD enhanced intracellular accumulation of cyclic AMP (cAMP) at the onset of stationary phase, resulting in the inhibition of caspase enzyme activity.

MATERIALS AND METHODS

Chemicals and reagents. *N*-acetyl-Asp-Glu-Val-Asp-*p*-Nitroanilide (Ac-DEVD-pNA) and biotinylated DEVD-CHO were purchased from Calbiochem. The biotin-conjugated, polyclonal anti-active human caspase-3 antibody; streptavidin agarose; streptavidin-horseradish peroxidase conjugate; and annexin V-fluorescein isothiocyanate (FITC) detection kit were obtained from PharMingen. Hybond-P, a hydrophobic polyvinylidene difluoride membrane, was procured from Amersham Pharmacia. Citrate lyase was from Fluka. Aldolase, glycerophosphate dehydrogenase, triosephosphate isomerase, fructose-6-phosphate, fructose-2, 6-diphosphate, forskolin, cAMP, caffeine, L and D forms of amino acids, ATP, sodium pyruvate, β -NADH, malic dehydrogenase, 4-chloro-1-naphthol (color reagent solution), and general assay reagents were purchased from Sigma Chemical Co.

Media and culture conditions. Five different liquid growth media were used, including starch minimal medium, M9 medium, Luria-Bertani (LB) medium, nutrient broth (NB) medium, and casein medium. Starch minimal medium (pH 6.8) contained starch (1%), $K_2HPO_4 \cdot 3H_2O$ (0.3%), KH_2PO_4 (0.15%), ammonium sulfate (0.2%), L-methionine (0.05%), nicotinic acid (0.025%), and L-glutamate (0.025%). M9 medium (pH 7.2) contained Na_2HPO_4 (0.6%), KH_2PO_4 (0.3%), NaCl (0.05%), NH_4Cl (0.1%), $MgSO_4 \cdot 7H_2O$ (1 mM), $CaCl_2$ (0.1 mM), and glucose (2%). LB medium (pH 7.0) contained tryptone (1%), yeast extract (0.5%), and NaCl (1%). NB medium (pH 7.4) contained peptic digest of animal tissue (0.5%), NaCl (0.5%), beef extract (0.15%), and yeast extract (0.15%). Casein medium (pH 7.0) contained peptone (0.5%), yeast extract (0.3%), casein (0.5%), and glycerol (2%). Tryptone water (pH 7.0) contained casein hydrolysate (1%) and NaCl (0.5%).

Inoculation was carried out by addition of a single isolated colony of *X. campestris* pv. *glycines* to the medium and was incubated for 24 h on a rotary

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shaker (150 rpm) at ambient temperature ($26 \pm 2^\circ\text{C}$), followed by further incubation under static conditions at ambient temperature. A culture grown for 24 h was incubated further under static conditions at the ambient temperature in order to observe RCD. For viable cell counts, aliquots of the culture broth were withdrawn and serially diluted using sterile saline (0.85%) and transferred to LB agar using a spread plate technique. Plates were incubated at $26 \pm 2^\circ\text{C}$ for 72 h. Viable cell counts were obtained at the end of the incubation period by counting colonies.

Growth profile on soybean leaves. Soybean seeds (cv. Moneta) were grown in pots in a plant growth chamber. At the third trifoliate leaf stage, the leaves were inoculated with different numbers of *X. campestris* pv. glycines cells. One milliliter of an overnight *X. campestris* pv. glycines culture grown in starch minimal medium was centrifuged ($10,000 \times g$ for 10 min), and the pellet was washed with saline (0.85%) and suspended in a 1-ml aliquot of the same. The suspension was serially diluted prior to inoculation. The leaves were lightly punctured, and aliquots (10 μl) of the suspension containing ca. 10^2 , 10^4 , and 10^6 CFU were placed on the punctured spots. After the desired incubation period, the inoculated leaves were removed and the inoculated spots were cut using sterile scissors, suspended, and macerated in saline for determination of viable cell counts on LB agar. Plates were incubated for 72 h at ambient temperature before counting.

Caspase-3 assay. A single colony of *X. campestris* pv. glycines was transferred to 10 ml of medium and incubated overnight (~ 18 h) on a rotary shaker (150 rpm) at ambient temperature. A 1-ml ($\sim 10^8$ CFU/ml) aliquot of the culture was centrifuged at $10,000 \times g$ for 10 min. The pellet was washed once with phosphate-buffered saline (PBS; 10 mM, pH 7.4) and suspended in 500 μl of caspase assay buffer containing HEPES (20 mM, pH 7.6), NaCl (100 mM), 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) (0.1%), dithiothreitol (DTT) (10 mM), EDTA (100 μM), and glycerol (10%). The cells were then lysed by freeze-thaw and sonication on ice for 15 s. Protein equivalent to 25 μg was mixed with 200 μM of synthetic colorimetric substrate, *N*-acetyl-Asp-Glu-Val-Asp-*p*-Nitroanilide (Ac-DEVD-pNA), prepared in dimethyl sulfoxide as a 10 mM stock, and incubated at 37°C for 30 min in 1 ml assay buffer (HEPES [20 mM, pH 7.6], NaCl [100 mM], CHAPS [0.1%], DTT [10 mM], EDTA [100 μM], and glycerol [10%]) (30, 43). After incubation, the absorbance at 405 nm was measured using a spectrophotometer. The protein concentration was determined by the standard Bradford method (4).

SDS-PAGE. Overnight-grown *X. campestris* pv. glycines cells were harvested by centrifuging at $10,000 \times g$ for 10 min, and the pellet washed twice with phosphate-buffered saline (PBS; 10 mM, pH 7.5) and suspended in sterile Milli-Q water. The cell suspension was mixed with an equal volume of 2 $\times$ gel loading buffer (Tris [100 mM, pH 6.8], sodium dodecyl sulfate [SDS] [4%], glycerol [20%], bromophenol blue [0.002%], and β -mercaptoethanol [200 mM]). The mixture was heated to 95°C for 10 min, immediately chilled on ice for 5 min, and centrifuged at $12,000 \times g$ for 10 min. A 50- μl aliquot of the supernatant was loaded on a 10% (wt/vol) Tris-glycine SDS-polyacrylamide slab gel, which was run vertically at a 35-mA constant current on a polyacrylamide gel electrophoresis (PAGE) system (Techno Source, India).

Western blotting. After completion of the SDS-PAGE run, electroblotting was performed using a Hybond-P membrane in a transfer buffer (25 mM Tris, 192 mM glycine [pH 8.3], 20% methanol) employing a 50-mA constant current overnight at 4°C . The blotted membrane was hybridized with 10 μl (0.5 mg/ml) of the affinity-purified biotin-conjugated polyclonal rabbit anti-active human caspase-3 antibody per the method described earlier (12). The hybridized caspase protein was subjected to secondary hybridization with 50 μl of streptavidin-horseradish peroxidase conjugate for 1.5 h. The blot was washed once with Tris-buffered saline (TBS)-Tween 20 (0.05%) and once with TBS for 5 min, respectively, and detected using color reagent solution (4-chloro-1-naphthol/ H_2O_2).

Paper chromatography. A single colony of *X. campestris* pv. glycines was inoculated in 20 ml of starch minimal medium and incubated on a rotary shaker (150 rpm) at ambient temperature ($26 \pm 2^\circ\text{C}$). An aliquot (1 ml) was withdrawn at different time intervals and centrifuged at $10,000 \times g$ for 10 min. Whatman paper no. 3 of the required size was saturated with the mobile phase and air dried. A 10- μl aliquot of the above-described supernatant was applied as a small spot 2 cm above the bottom edge and air dried. Similarly, 2- μl aliquots of the standards (1 M) glucose, maltose, dextrin, and soluble starch were also spotted. *N*-butanol/ethanol/water were mixed in the proportion of 52:33:15 and shaken well, and phases were allowed to separate. The upper organic phase was withdrawn, passed through a filter paper, and transferred to the chromatographic tank. The tank was kept tightly closed for 3 to 4 h to ensure saturation with the mobile phase. The spotted paper was placed in the tank with the bottom edge carefully dipped in the solvent. After a 6-h run, the paper was removed and the

solvent front was marked with a pencil, and the paper was air dried for 15 min. For spot detection, the paper was dipped in 200 ml acetone containing 1 ml of a saturated solution of AgNO_3 for 1 min. The paper was further dipped in 200 ml of NaOH (0.5 N) for 2 min. As the spots developed, the paper was taken out of the solution and further dipped in 200 ml 5% sodium thiosulfate to stop the reaction and clear the background.

cAMP assay. A cAMP enzyme immunoassay kit (Sigma CA-200) was used to determine intracellular levels of cAMP. *X. campestris* pv. glycines cells were harvested by centrifugation ($10,000 \times g$ for 10 min) and washed once with PBS, and the pellet was suspended in 500 μl 0.1 M HCl. The cells ($\sim 7 \times 10^8$ CFU/ml) were lysed by freeze-thaw and sonication on ice for 15 s and centrifuged at $6,000 \times g$ at ambient temperature, and the supernatant used directly in the assay. An aliquot (200 μl) was acetylated with acetylating reagent. The acetylated samples (100 μl) were aliquoted into a 96-well plate, neutralized with the neutralizing reagent, and treated with cAMP conjugate and cAMP antibody as per the instructions of the manufacturer. After incubation at ambient temperature for 2 h, the wells were aspirated and washed thrice with wash solution, followed by treatment with substrate and incubation at ambient temperature for 1 h. The reaction was stopped with stop solution, and the absorbance was read at 405 nm in a universal microplate reader (Bio-Tek Instruments). Each assay was performed independently in triplicate, and the results were analyzed as per the instructions provided by the manufacturer (Sigma Chemical Co., St. Louis, Mo.).

Pyruvate assay. *X. campestris* pv. glycines cells were harvested by centrifugation ($10,000 \times g$ for 10 min) and washed once with PBS. The cells ($\sim 7 \times 10^8$ CFU/ml) were lysed in 2 ml of perchloric acid (PCA; 8%) and centrifuged at $6,000 \times g$ at ambient temperature, and the supernatant used directly in the assay. Intracellular pyruvate concentrations were determined with a commercial kit (Sigma 726-UV). In this assay, the oxidation of NADH was monitored at 340 nm after the NADH-linked conversion of pyruvate to lactate by lactate dehydrogenase. The perchloric acid (PCA) supernatant was used directly for the assay in a cuvette (3 ml). The volume of PCA supernatant (2 ml) was brought to 3 ml by the addition of 500 μl of NADH (0.5 mg/ml) in Trizma base solution (1.5 M) and 500 μl of free Trizma base solution (1.5 M). The initial absorbance (340 nm) was recorded versus that of water as a reference. The reaction was initiated by the addition of 50 μl of lactate dehydrogenase (1 KU/ml), and the final absorbance (340 nm) was recorded after incubation at ambient temperature for 5 min. The change in absorbance was determined for each sample, and the results were analyzed as instructed by the manufacturer (Sigma Chemical Co., St. Louis, Mo.).

Estimation of citrate. *X. campestris* pv. glycines cells were harvested by centrifugation ($10,000 \times g$ for 10 min) and washed once with PBS. The cells ($\sim 7 \times 10^8$ CFU/ml) were lysed by adding 0.5 M perchloric acid. The resultant supernatant was neutralized with 1 M potassium hydroxide and passed through a 0.45- μm filter. An aliquot of the filtrate (100 μl) was used for the enzymatic estimation of citrate as described previously (7, 44). The decline in NADH concentration was directly proportional to the amount of citrate present in the sample, and the results were represented as percent reduction of A_{340} from the initial reading.

Flow cytometric assay. *X. campestris* pv. glycines cells grown for different time periods under different growth conditions were harvested by centrifugation at $10,000 \times g$ for 10 min, washed twice with ice-cold PBS2, and resuspended in 250 μl of PBS2. An aliquot (50 μl) of the above suspension was mixed with 900 μl annexin V-FITC binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl_2), 5 μl annexin V-FITC (PharMingen) and incubated for 15 min at ambient temperature ($26 \pm 2^\circ\text{C}$). The cells were treated with 5 μl of propidium iodide (PI) (50 $\mu\text{g}/\text{ml}$) prior to acquisition on flow cytometry for 10,000 events (FACS Vantage; Becton Dickinson).

PFK assay. Cells at the onset of the stationary phase were harvested, washed once with PBS, and lysed by sonication in 50 mM potassium phosphate buffer [50 mM $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ (pH 7.4), 5 mM DTT, 5 mM $(\text{NH}_4)_2\text{SO}_4$, 0.5 mM phenylmethylsulfonyl fluoride]. The suspension was centrifuged at $6,000 \times g$ for 20 min, and the cell extract was used directly for the assay. The phosphofructokinase (PFK) activity was determined spectrophotometrically using a coupled enzyme assay as described previously (37) with minor modifications. The reaction was performed in 100 mM imidazole-HCl (pH 7.2) buffer containing an additional 5 mM $(\text{NH}_4)_2\text{SO}_4$, and the PFK activity is represented as percent loss of NADH in the reaction at A_{340} .

Nucleotide sequence accession number. The polysaccharide deacetylase gene from *X. campestris* pv. glycines cloned and sequenced in the course of this work has been deposited in GenBank under accession no. DQ394570.

FIG. 1. Growth profile of *X. campestris* pv. *glycines* on soybean plants. Aliquots (10 μ l) containing 10² (▲), 10⁴ (●), and 10⁶ (◻) CFU were inoculated on soybean leaves, and the cell numbers were determined at 24, 48, 96, and 120 h from the start of inoculation. Each point on the graph is the mean value from three independent determinants ($n = 3$).

RESULTS

In vivo studies on soybean leaves. The growth profile of *X. campestris* pv. *glycines* on leaves of its host plant soybean (*Glycine max*) is illustrated in Fig. 1. Aliquots (10 μ l) containing 10², 10⁴, and 10⁶ CFU were placed on the lightly punctured spots on the leaves. An initial inoculum of 10² CFU/spot yielded approximately 10⁵ CFU/spot after 48 h of incubation, whereas the higher inocula of 10⁴ and 10⁶ CFU/spot yielded 10⁶ and 10⁷ CFU/spot, respectively (Fig. 1). No reduction in cell number on these spots was observed in the next 120 h (Fig. 1). Thus, *X. campestris* pv. *glycines* cells were not only able to

FIG. 2. Western blot showing expression of caspase-3-like protein in *X. campestris* pv. *glycines* cells grown in starch minimal, M9, nutrient broth, LB, casein, yeast extract (YE), and tryptone media. Protein equivalents of 100 μ l overnight-grown culture were loaded into each well and subjected to Western blotting with polyclonal rabbit anti-active human caspase-3 antibody. The antibody is known to react with both the unprocessed procaspase (upper band) and the larger subunit of the active caspase (lower band) (B.D. PharMingen Technical Data Sheet, catalog no. 556425).

grow well on the leaves of soybean but also maintained their viability without undergoing RCD.

Survival of *Xanthomonas* in different media. Viable cell counts in stationary-phase cultures of *X. campestris* pv. *glycines* grown for 24 and 96 h in different culture media are shown in Table 1. In media such as LB, NB, and casein, rapid declines in the viable cell numbers were observed between 24 and 96 h of incubation. The cell count was reduced from 8 log₁₀ CFU/ml at the onset of the stationary phase (24 h after the start of incubation) to 3 log₁₀ CFU/ml at the end (72 h from the onset of stationary phase) in both LB and NB media and to almost 0 in casein medium (Table 1). Similarly, the major constituents of LB medium such as yeast extract and tryptone (casein enzymatic hydrolysate) were also tested for their effects on cell viability during incubation in stationary phase. In yeast extract the cell count was reduced from 8 log₁₀ CFU/ml at the beginning to 4 log₁₀ CFU/ml, and in tryptone it was reduced to 5 log₁₀ CFU/ml at the end of the incubation (Table 1). On the other hand, the viable cell numbers in starch minimal and M9 media remained stable (~8 log₁₀ CFU/ml) both at the beginning and at the end of the incubation in stationary phase. The occurrence of RCD in nutrient-rich medium suggested that a peptide(s) or amino acid(s) might be an inducer(s) of RCD, while the starch and M9 media appeared to lack such an inducer(s).

Caspase expression profile in different growth media. Extracts from cells grown in the nutrient-rich LB, NB, casein, yeast extract, and tryptone media that induced RCD displayed strong hybridization signals with the human caspase-3 antibody (Fig. 2). However, in extracts from cells grown in starch minimal and M9 media, the presence of caspase-3-like protein was negligible. Protein extracted from *X. campestris* pv. *glycines* cells grown on its natural host, soybean, did not show the presence of caspase-3-like protein (data not shown).

Amino acids as inducers of RCD. Since RCD was observed in *Xanthomonas* only in nutrient-rich media, amino acids were screened as the possible inducers of this phenomenon. All the 20 standard amino acids were tested for induction of RCD by their addition to starch minimal medium in which *X. campestris* pv. *glycines* did not display RCD. Among these amino acids,

TABLE 1. Viable cell counts of *X. campestris* pv. *glycines* at 24 and 96 h of stationary-phase incubation in different media^a

Medium	Viable cell count (CFU/ml) at:	
	24 h	96 h
Starch	4 × 10 ⁸ ± 2 × 10 ⁸	4 × 10 ⁸ ± 1 × 10 ⁸
M9 minimal	6 × 10 ⁸ ± 1 × 10 ⁸	3 × 10 ⁸ ± 1 × 10 ⁸
Luria-Bertani	3 × 10 ⁹ ± 1 × 10 ⁹	4 × 10 ³ ± 3 × 10 ³
Nutrient broth	2 × 10 ⁸ ± 1 × 10 ⁸	4 × 10 ³ ± 3 × 10 ³
Casein	2 × 10 ⁸ ± 2 × 10 ⁸	0 ± 0
Yeast extract	5 × 10 ⁸ ± 1 × 10 ⁸	6 × 10 ⁴ ± 3 × 10 ⁴
Tryptone	4 × 10 ⁸ ± 1 × 10 ⁸	7 × 10 ⁵ ± 2 × 10 ⁵
Starch + L-Ala (80 mM)	2 × 10 ⁹ ± 9 × 10 ⁷	7 × 10 ⁵ ± 2 × 10 ⁵
Starch + Gly (80 mM)	1 × 10 ⁹ ± 1 × 10 ⁸	9 × 10 ⁴ ± 6 × 10 ⁴
Starch + D-Thr (50 mM)	4 × 10 ⁸ ± 9 × 10 ⁷	1 × 10 ⁴ ± 1 × 10 ³
Starch + D-Val (50 mM)	1 × 10 ⁸ ± 2 × 10 ⁷	1 × 10 ⁴ ± 1 × 10 ⁴
Starch + D-Met (50 mM)	1 × 10 ⁸ ± 6 × 10 ⁷	3 × 10 ³ ± 1 × 10 ³
Starch + acetate (100 mM)	1 × 10 ⁹ ± 2 × 10 ⁸	2 × 10 ⁹ ± 1 × 10 ⁹
Starch + Pyr (75 mM)	8 × 10 ⁸ ± 1 × 10 ⁸	2 × 10 ⁶ ± 7 × 10 ⁵
Starch + citrate (25 mM)	1 × 10 ⁹ ± 7 × 10 ⁸	7 × 10 ⁵ ± 2 × 10 ⁵
Starch + succinate (100 mM)	2 × 10 ⁹ ± 1 × 10 ⁹	9 × 10 ⁶ ± 6 × 10 ⁵
Starch + malate (100 mM)	1 × 10 ⁹ ± 6 × 10 ⁸	8 × 10 ⁵ ± 1 × 10 ⁵
LB + D-Glu (2%)	1 × 10 ⁹ ± 6 × 10 ⁸	1 × 10 ⁹ ± 9 × 10 ⁹
LB + cAMP (1 mM)	6 × 10 ⁸ ± 4 × 10 ⁸	7 × 10 ⁷ ± 1 × 10 ⁷
LB + caffeine (1 mM)	3 × 10 ⁸ ± 4 × 10 ⁷	1 × 10 ⁸ ± 9 × 10 ⁶
LB + forskolin (30 μ M)	2 × 10 ⁸ ± 4 × 10 ⁷	3 × 10 ⁸ ± 1 × 10 ⁷

^a Figures are rounded to the nearest whole number. Results are given $\pm$ standard deviations.

FIG. 3. Western blot showing induction of caspase synthesis in the cells grown in starch minimal medium containing inducer amino acids D-Met (50 mM), D-Thr (60 mM), D-Val (50 mM), Gly (80 mM), L-Ala (80 mM), and control (Cont; without addition). Protein equivalents of 200 μ l overnight-grown culture were loaded into each well and subjected to Western blotting with polyclonal rabbit human anti-active caspase-3 antibody. The antibody is known to react with unprocessed procaspase (upper band) and the larger subunit of the active caspase (lower band) (B.D. PharMingen Technical Data Sheet, catalog no. 556425).

only glycine and L-alanine (80 to 100 mM) were found to induce RCD, as indicated by the decline in the stationary-phase cell numbers (Table 1). Interestingly, the D isomers of valine, methionine, and threonine (50 to 60 mM) were also found to induce RCD (Table 1). The viable cell count at the end of 96 h of incubation with these D amino acids was found to be reduced to around 3 to 4 log₁₀ CFU/ml. These amino acids could induce RCD only when added to the culture at an early stage (at 6 h or before) of growth (data not shown). Importantly, the induction of RCD by the above amino acids was accompanied by the appearance of caspase enzyme activity in cells (data not shown), and the presence of caspase-3-like protein was confirmed by Western blot analysis (Fig. 3).

Effect of pyruvate and TCA metabolites on RCD. Since glycine and L-alanine are pyruvate-generating amino acids, it was interesting to test the effect of pyruvate and other tricarboxylic acid (TCA) metabolites on RCD. Table 1 shows the cell viability of the *X. campestris* pv. *glycines* cells grown in starch medium with added pyruvate, malate, citrate, and succinate. Citrate (25 mM), pyruvate (75 mM), and malate (100 mM) were found to induce RCD, as is evident from the reduction in stationary-phase cell numbers (Table 1). Among the metabolites (Table 1) tested, citrate at 25 mM appeared to be the most effective molecule in promoting RCD.

Acetate was not able to induce RCD in *X. campestris* pv. *glycines* (Table 1). Moreover, no pyruvate oxidase activity was detected in *X. campestris* pv. *glycines* cells grown in LB and starch media or in starch medium with added pyruvate. The pH profile of the supernatant of *X. campestris* pv. *glycines* cultures did not indicate any accumulation of acids. On the contrary, the pH of the LB-grown cultures showed an increase from 7.1 to 7.9. Even in starch medium or starch medium with

FIG. 4. pH profile of the culture supernatant at different time points (6 h to 120 h). Culture pH of starch-grown cells (●), cells grown in citrate fortified starch medium (▲), and the culture supernatant of LB-grown cells (○). Each point on the graph is the mean pH of the samples in triplicate.

added citrate, the pH of the culture supernatant remained between 6.7 and 6.8 (Fig. 4).

Induction of caspase-3 synthesis by pyruvate and TCA metabolites. Though all four metabolites showed increased expression of caspase-3-like protein compared to that of the starch-grown controls, citrate (25 mM) seemed to be the most effective inducer (Table 2). Addition of isocitrate, α -ketoglutaric acid (α -KG), fumarate, and oxaloacetate to starch medium resulted in neither a reduction of stationary-phase cell number nor the expression of significant caspase-3-like protein, even at 100 mM concentration (Tables 1 and 2).

Table 2 shows the assay of caspase-3 activity in cultures

TABLE 2. Relative band intensity measured from Western blot and caspase activity for *X. campestris* pv. *glycines* cells treated with different metabolites (0 to 100 mM)

Metabolite	% Relative band intensity ^b	Caspase activity ^c ($\pm$ SD)
Control (wild-type <i>X. campestris</i> pv. <i>glycines</i> AM2 in LB) ^a	100 (100)	0.174 $\pm$ 0.005
Pyruvate (75 mM)	90 (73.9)	0.127 $\pm$ 0.004
Citrate (75 mM)	78.4 (82)	0.152 $\pm$ 0.004
Citrate (50 mM)	91 (94)	0.151 $\pm$ 0.003
Citrate (25 mM)	51.3 (81)	0.126 $\pm$ 0.005
Malate (100 mM)	82.6 (48)	0.119 $\pm$ 0.002
Isocitrate (100 mM)	9	0.049 $\pm$ 0.001
α -KG (100 mM)	7.4	0.046 $\pm$ 0.002
Succinate (100 mM)	39 (11)	0.063 $\pm$ 0.002
Starch	5.1	0.047 $\pm$ 0.002

^a Wild-type cells *X. campestris* pv. *glycines* AM2 (Control) grown in LB without addition.

^b Relative band intensity of the active, unprocessed caspase and its larger subunit (values in parenthesis) as measured from the Western blotting and expressed in percentages (Syngene bio-imaging system; United Kingdom). Control band intensity was set at 100%. Protein equivalents of 200 μ l overnight-grown culture were loaded into each well and subjected to Western blotting with polyclonal rabbit human anti-active caspase-3 antibody.

^c Twenty-five micrograms of the total protein was used for each assay with the colorimetric caspase-3 substrate (Ac-DEVD-pNA), and the absorbance (A_{405}) was recorded after 30 min.

TABLE 3. Relative band intensity from Western blot and caspase activity of wild-type and mutant *X. campestris* pv. *glycines* strains

Strain	% Relative band intensity ^b	Caspase activity ^c (±SD)
Wild type (<i>X. campestris</i> pv. <i>glycines</i> AM2) ^a	100	0.154 ± 0.003
M-11	21	0.063 ± 0.001
M-20	7.25	0.05 ± 0.002
M-42	4	0.040 ± 0.005

^a Control wild-type cells (*X. campestris* pv. *glycines* AM2) were grown in starch medium fortified with citrate (50 mM).

^b Relative band intensities of the active, unprocessed caspase as measured from Western blots and expressed in percentages. Control band intensity was set at 100%. Protein equivalents of 200 µl overnight-grown culture were loaded into each well and subjected to Western blotting with polyclonal rabbit human anti-active caspase-3 antibody.

^c Twenty micrograms of the total protein was used for each assay with the caspase-3 substrate (Ac-DEVD-pNA), and the absorbance (A_{405}) was recorded after 30 min.

treated with the above-mentioned metabolites. Addition of citrate (25 mM) and pyruvate (75 mM) to starch medium-grown cultures resulted in a nearly threefold increase in caspase-3 activity. Malate (100 mM) also resulted in a significant increase in the activity compared to that of the starch-grown control cells without the addition.

Absence of RCD in caspase mutants. In order to confirm that caspase was induced by pyruvate, three *X. campestris* pv. *glycines* caspase mutants, M-11, M-20, and M-42, described in an earlier study (8), were also tested for rapid cell death, caspase biosynthesis, and caspase enzyme activity. As can be seen in Table 3, neither caspase protein nor activity was observed when the mutants were grown in starch medium with 50 mM citrate. None of the mutants expressed RCD under the same conditions (data not shown).

Accumulation of pyruvate and citrate in cells undergoing RCD. In LB- and starch-grown cells with added glycine or L-alanine, higher levels of intracellular pyruvate were detected compared to those in cells grown in starch medium without the inducer amino acids (Fig. 5A). Similarly, a high intracellular concentration of citrate was observed when the cells were grown in starch medium fortified with 80 mM L-alanine or pyruvate (Fig. 5B). Significant citrate levels were also observed in LB-grown cells (Fig. 5B), implying that the addition of glycine or L-alanine led to intracellular buildup of pyruvate and citrate that triggered RCD in *X. campestris* pv. *glycines*.

PFK has no role in RCD. Citrate at high concentrations is known to be a potent allosteric inhibitor of phosphofructokinase (PFK), a key enzyme in glycolysis. In our studies, *in vivo* PFK activity was found to remain unaffected under conditions that promoted citrate accumulation (Fig. 6).

Effect of starch hydrolysis products on RCD. Paper chromatograms revealed the presence of starch hydrolysis products present in the culture supernatant of *X. campestris* pv. *glycines* cells grown in starch minimal medium (data not shown). After 24 h of incubation, all three hydrolytic products of starch, namely, dextrin, maltose, and glucose, were detected in the culture supernatant. At 48 h, all three hydrolytic products were found to be present; however, on further incubation (72 h or more), all the partially hydrolyzed products were completely converted to glucose (data not shown). LB medium fortified

with 5% starch is known to inhibit caspase biosynthesis and RCD (12). The components of starch such as maltose (5%) and dextrin (4%) were also found to inhibit caspase biosynthesis and thereby RCD (Table 4). Glucose, being the end product of starch hydrolysis, was further tested for its effect on the RCD. Addition of 2% glucose to *X. campestris* pv. *glycines* cultures growing in LB medium was found to completely inhibit the RCD process but not caspase biosynthesis, even at 5% concentration (Tables 1 and 4).

Inhibition of RCD by cAMP and its modulators. Cyclic AMP, known to play a key role in the regulation of many

FIG. 5. A. Intracellular pyruvate in *X. campestris* pv. *glycines* grown in starch medium, LB medium, and starch medium supplemented with the RCD inducers L-alanine (80 mM) and pyruvate (80 mM). One milliliter of a culture grown for 24 h (onset of stationary phase) was utilized for each assay. Intracellular pyruvate concentrations are represented in milligrams/deciliter. Each experiment was performed twice in triplicate, and results of one typical experiment are represented here ($n = 3$). Error bars indicate standard deviations (SD). B. Intracellular citrate concentration in *X. campestris* pv. *glycines* grown in LB medium, starch medium, and starch medium supplemented with RCD inducers (L-alanine [80 mM] and pyruvate [80 mM]). One milliliter of a culture grown for 24 h (onset of stationary phase) was utilized for each assay, and intracellular citrate was represented as percent loss of NADH in the reaction as measured by reduction in A_{340} . Each experiment was performed twice in triplicate, and the results of one typical experiment are represented here ($n = 3$). Error bars indicate SD.

FIG. 6. PFK activity of *X. campestris* pv. glycines cells grown under different growth conditions: LB medium, starch medium, and starch medium fortified with 50 mM citrate (Starch+Cit). The PFK activity was represented as percent loss of NADH in the reaction as measured by reduction in A_{340} . Approximately 10^6 cells were used for each assay. Each bar represents means and standard deviations of quadruplicate determinations ($n = 4$). Error bars indicate standard deviations.

enzymes involved in diverse cellular functions in eukaryotes (34), was found to significantly inhibit the RCD process at around 1 to 1.5 mM without inhibiting caspase biosynthesis (Tables 1 and 4). Further, caffeine, known to inhibit the enzyme phosphodiesterase, which breaks down cAMP (21), was also found to significantly inhibit the RCD in *X. campestris* pv. glycines at 1 mM (Table 1). Forskolin, an extract of the herb *Coleus forskohlii*, known to activate adenylate cyclase that helps increase the levels of cAMP inside the cell (28, 38, 47), was also found to completely inhibit the RCD at a concentration of 30 μ M (Table 1). Approximately 65% inhibition of in vivo caspase activity was observed in the presence of RCD inhibitors such as glucose, cAMP, caffeine, and forskolin (Table 4).

cAMP accumulation inhibited RCD. Figure 7A shows the intracellular cAMP levels in cells grown in starch medium at

FIG. 7. A. Intracellular cAMP levels in *X. campestris* pv. glycines cells grown under different conditions, starch medium (control), starch medium with pyruvate (80 mM), and starch medium with D-threonine (60 mM), after 15 h (mid-log phase), 24 h (onset of stationary phase), and 42 h (stationary phase). Intracellular cAMP levels were represented as picomoles/milliliter against time. The data are the means and standard deviations of three independent determinations ($n = 3$) (starch control means at different time points were statistically significant from the two treatment means [pyruvate and D-threonine] as observed by simple one-way analysis of variance, with $P < 0.01$). Error bars indicate standard deviations. B. Intracellular cAMP levels in *X. campestris* pv. glycines cells grown under different conditions, LB medium (control), LB with caffeine (2.5 mM), LB with glucose (2%), and LB with forskolin (40 μ M), after 15 h (mid-log phase), 24 h (onset of stationary phase), and 42 h (stationary phase). No value assigned for LB culture grown for 42 h as the net absorbance (A_{405}) value was much below the minimum sensitivity of the intracellular cAMP immunoassay kit (0.078). Intracellular cAMP levels were represented as picomoles/milliliter against time for different growth conditions. The data are the means and standard deviations of at least three independent determinations ($n = 3$). (Treatment means are statistically significant from the control means [LB] as observed by simple analysis of variance, with $P < 0.5$.) Error bars indicate standard deviations.

TABLE 4. Relative band intensity from Western blot and caspase activity of *X. campestris* pv. glycines grown in LB fortified with different RCD inhibitors

Medium and RCD inhibitor	% Relative band intensity ^a	In vivo caspase activity ^b ($\pm$ SD)
LB (control)	100	0.165 $\pm$ 0.005
LB + glucose (2%)	92	0.063 $\pm$ 0.002
LB + cAMP (1 mM)	96	0.065 $\pm$ 0.005
LB + caffeine (2.5 mM)	99	0.060 $\pm$ 0.003
LB + forskolin (40 μ M)	100	0.061 $\pm$ 0.005
LB + maltose (5%)	28.4	0.054 $\pm$ 0.004
LB + dextrin (4%)	8.7	0.051 $\pm$ 0.003

^a Relative band intensities of the active, unprocessed caspase measured from the Western blots and expressed in percentages. Control band intensity was set at 100%. Protein equivalents of 100 μ l overnight-grown culture were loaded into each well and subjected to Western blotting with polyclonal rabbit human anti-active caspase-3 antibody.

^b Twenty micrograms of the total protein was used for each assay with the colorimetric caspase-3 substrate (Ac-DEVD-pNA), and absorbance (A_{405}) was recorded after 30 min.

different time points: 15 h (mid-log phase), 24 h (onset of stationary phase), and 42 h (stationary phase). The mean cAMP concentrations during these time periods were found to be 0.8, 1.1, and 0.42 pmol/ml, respectively, whereas starch medium-grown cells fortified with RCD-promoting molecules

FIG. 8. RCD in *X. campestris* pv. *glycines* cells as assessed by flow cytometry-based annexin V-FITC assay at an excitation wavelength of 488 nm and emission wavelength of 520 nm. Shown are LB control, LB with D-glu (2%), and LB with caffeine (2.5 mM) 24 h after the start of incubation. Starch control and starch plus pyruvate (80 mM) (S+ Pyr) after 24 h and 65 h of the start of incubation are also shown. An aliquot (200 μ l) from 1 ml grown culture was taken for each assay, and each bar represents means of triplicate samples ($n = 3$). Error bars indicate standard deviations.

such as pyruvate (80 mM) and D-threonine (60 mM) were found to have reduced cAMP levels of less than 0.2 pmol/ml during mid-log (12 h) and stationary phase (42 h). Figure 7B shows the intracellular cAMP levels in *X. campestris* pv. *glycines* cells grown in LB medium and the same fortified with the inhibitors of RCD. Intracellular cAMP levels in LB-grown cells at 15, 24, and 42 h of incubation were 0.3, 0.6, and <0.078 pmol/ml, respectively (Fig. 7B). Caffeine (2.5 mM) and glucose (2%) resulted in increased cAMP levels to more than 2 pmol/ml at the onset of stationary phase, while the presence of forskolin (40 μ M) led to moderate enhancement of cAMP levels to above 1 pmol/ml (Fig. 7B). Cyclic AMP levels were found to be always high at the onset of stationary phase (24 h) in both starch- and LB-grown cells (Fig. 7). Thus, RCD inhibitors (glucose, caffeine, and forskolin) were found to enhance intracellular cAMP, while the inducers of RCD reduced cAMP accumulation in *X. campestris* pv. *glycines*.

RCD is accompanied by externalization of phosphatidyl serine. Apart from induction of active caspase-3 synthesis, RCD in *X. campestris* pv. *glycines* was accompanied by externalization of phosphatidyl serine, another feature of eukaryotic PCD (23). Flow cytometric analysis using annexin V-FITC and propidium iodide (PI) labeling were carried out on *X. campestris* pv. *glycines* grown in different media with the inducers and inhibitors of RCD. In control starch-grown cultures, approximately 3 to 4% of the cells were found to be annexin-V positive. This population increased to around 38% and 78% in the presence of pyruvate after 24 and 65 h of the start of incubation, respectively (Fig. 8). Conversely, in LB medium after 24 h of incubation, 30 to 32% of the cell population was found to be annexin-V positive, which was reduced to 6 to 6.5% in cultures with the addition of glucose and to 3 to 3.5% with caffeine (Fig. 8). The percentage of PI-positive cells was found to be negligible (less than 4%) in all cells.

DISCUSSION

The viability of microorganisms is known to be affected by the medium in which they are cultivated and by conditions of storage. This often poses problems in their maintenance both in the laboratory and in the culture collection centers. We experienced similar problems with *X. campestris* pv. *glycines*. It was observed that the *X. campestris* pv. *glycines* cultures were quite stable over a period of time in starch medium but not in LB or other protein-rich media. This led to the finding that *X. campestris* pv. *glycines* undergoes RCD in the postexponential stationary phase in nutrient-rich media (10–14). Stationary-phase cell death in bacteria has traditionally been described as autolysis. The term has also been used in the context of eukaryotic cell lysis. Cell autolysis is normally understood to be the result of hydrolytic enzyme activity that is released from lysosomes or equivalent organelles in cells. However, it is clear from our studies that the observed RCD in *X. campestris* pv. *glycines* was different from autolysis. It is programmed at an early phase of bacterial growth (<6 h from the start of incubation) in response to medium constituents and their concentrations. This is evident from the fact that synthesis of the caspase protein in *X. campestris* pv. *glycines* was initiated in LB medium around 4 to 6 h after inoculation but not in the starch medium (11, 12). Patton et al. (31) recently proposed that cell death in *Staphylococcus aureus* may occur due to accumulation of acetic acid in the stationary phase as a result of the induction of the *cid* genes responsible for the oxidation of pyruvate. Results from our studies showed that acetic acid induced neither cell death nor the synthesis of caspase protein in *X. campestris* pv. *glycines*. Thus, acetic acid seems to have no role in the observed RCD of the *X. campestris* pv. *glycines* cultures. We also failed to detect pyruvate oxidase activity in *X. campestris* pv. *glycines* under conditions that promote RCD. Moreover, analysis of the genome sequence of *Xanthomonas* has not revealed the presence of even a distant homologue of a pyruvate oxidase.

Glycine and L-alanine were found to induce synthesis of active caspase-3-like protein and consequently RCD in *X. campestris* pv. *glycines*. It was interesting to note that D isomers of amino acids were capable of inducing RCD. Though D amino acids are known to occur in bacteria, their exact role in cell physiology still remains unclear (25). Since the inducer amino acids were essentially pyruvate generating, the effect of pyruvate on the RCD induction was studied. The results revealed that cells undergoing RCD accumulated pyruvate as well as citrate. As relatively lower concentrations of citrate (25 mM) were required to induce RCD, it appeared to be a more effective trigger for the synthesis of active caspase-3-like protein and resultant RCD in *X. campestris* pv. *glycines*. In order to substantiate further the relationship between RCD and pyruvate metabolism, studies were carried out in modified starch medium in which the starch component was replaced with citrate (25 mM). The results observed here were similar to those for the starch-grown cells with added citrate. Moreover, *in vivo* PFK activity was unaltered in the presence of citrate. The role of citrate in the induction of caspase-3 synthesis and RCD remains to be elucidated.

In microorganisms, high glucose concentrations are known to reduce cellular cAMP levels, resulting in catabolite repres-

sion. However, contrary to expectations, we observed higher cellular cAMP levels in the presence of inhibitors of RCD including glucose and lower cAMP levels in the presence of inducers of RCD. That the buildup of cAMP indeed prevented RCD in *X. campestris* pv. *glycines* is further supported by the results observed with caffeine and forskolin. On the other hand, in certain eukaryotes, such as yeasts, higher glucose levels have been reported to result in higher cAMP levels (42). Whether *Xanthomonas* has a system similar to that of yeast in this respect remains to be clarified.

While caspase-deficient mutants synthesized limited amounts of caspase-3-like protein, significant caspase enzyme activity was not detected and the characteristic RCD observed in the wild type was not displayed (12). In this study, the mutants also failed to respond to the inducers of RCD. This clearly indicated that the genetic regulation of RCD in *X. campestris* pv. *glycines* correlated positively with the caspase-3-like activity.

Results of flow cytometric studies indicated a lack of significant uptake of propidium iodide and enhanced uptake of the annexin V-FITC label in the cells undergoing RCD, which suggested further that the nature of RCD in *X. campestris* pv. *glycines* is a type of programmed cell death (PCD). Annexin V is a 35- to 36-kDa Ca^{2+} -dependent phospholipid binding protein with high affinity to phosphatidyl serine, which explains its binding to membrane with exposed phosphatidyl serine. Annexin V binding indicated the externalization of phosphatidyl serine moieties in cells undergoing PCD (46). Changes in plasma membrane are reported to be one of the earliest features of apoptotic transformation in eukaryotes (23, 46).

Psi BLAST search with yeast caspase showed the presence of metacaspases in plants, fungi, protozoa, and bacteria that possess a conserved catalytic "cysteine-histidine" dyad of human caspases and constitute new members of a conserved superfamily of caspase-related proteases (1, 2). Among the putative caspase genes reported so far in the members of *Xanthomonadaceae* (<http://supfam.org>) (22), a polysaccharide deacetylase gene is interesting. It was picked up during a Psi BLAST search with yeast caspase (accession no. NP_014840) and possessed a characteristic "cysteine-histidine" dyad of human caspases. Hence, we have cloned and sequenced this particular gene from *X. campestris* pv. *glycines*. At the protein level, this gene exhibited similarity at the "cysteine-histidine" dyad with the members of *Xanthomonadaceae*, *Anabaena*, and the metacaspases of fungi.

The involvement of certain cellular metabolites such as pyruvate, citrate, and cAMP in the modulation of nutritional stress-related rapid cell death in *X. campestris* pv. *glycines* is reported here for the first time, reaffirming our earlier contention that this death was indeed programmed and genetically regulated.

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REFERENCES

- Anthony, G. U., K. O'Rourke, L. Aravind, M. T. Pisabarro, S. Seshagiri, E. V. Koonin, and V. M. Dixit. 2000. Identification of paracaspases and metacaspases two ancient families of caspase-like proteins, one of which plays a key role in MALT lymphoma. *Mol. Cell* 6:961-967.
- Aravind, L., and E. V. Koonin. 2002. Classification of the caspase-hemoglobinase fold: detection of new families and implications for the origin of the eukaryotic separins. *Proteins* 46:355-367.
- Bayles, K. W. 2003. Are the molecular strategies that control apoptosis conserved in bacteria? *Trends Microbiol.* 11:306-311.
- Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
- Cherny, I., and E. Gazit. 2004. The YefM antitoxin defines a family of natively unfolded proteins: implications as a novel antibacterial target. *J. Biol. Chem.* 279:8252-8261.
- Christensen, S. K., G. Maenhaut-Michel, N. Mine, S. Gottesman, K. Gerdes, and L. Van Melderen. 2004. Overproduction of the Lon protease triggers inhibition of translation in *Escherichia coli*: involvement of the YefM-YoeB toxin-antitoxin system. *Mol. Microbiol.* 51:1705-1717.
- Delhaize, E., P. R. Ryan, and P. J. Randall. 1993. Aluminum tolerance in wheat (*Triticum aestivum* L.) (II. Aluminum-stimulated excretion of malic acid from root apices). *Plant Physiol.* 103:695-702.
- Dragovich, T., C. M. Rudin, and C. B. Thomson. 1998. Signal transduction pathways that regulate cell survival and cell death. *Oncogene* 17:3207-3213.
- Engelberg-Kulka, H., and G. Glaser. 1999. Addition Modules and programmed cell death and antideath in bacterial cultures. *Annu. Rev. Microbiol.* 53:43-70.
- Gautam, S. 2003. Ph.D. thesis. University of Mumbai, India.
- Gautam, S., and A. Sharma. 2002a. Rapid cell death in *Xanthomonas campestris* pv. *glycines*. *J. Gen. Appl. Microbiol.* 48:67-76.
- Gautam, S., and A. Sharma. 2002b. Involvement of caspase-3 like protein in rapid cell death of *Xanthomonas*. *Mol. Microbiol.* 44:393-401.
- Gautam, S., A. Sharma, and I. Kobayashi. 2005. Programmed cell death in microorganisms. In M. Yamada (ed.), *Survival and death in bacteria*. Research Sign Post Press, India.
- Gautam, S., and A. Sharma. 2005. Programmed cell death: an overview, p. 122-157. In C. Chakraborty (ed.), *Advances in biochemistry and biotechnology*. Daya Publishing House, India.
- Gerdes, K., L. K. Poulsen, A. K. Thisted, J. M. Nielsen, and P. H. Andreassen. 1990. The *hok* killer gene family in gram-negative bacteria. *New Biol.* 2:946-956.
- Goffredsen, M., and K. Gerdes. 1998. The *Escherichia coli relBE* genes belong to a new toxin-antitoxin gene family. *Mol. Microbiol.* 29:1065-1076.
- Grady, R., and F. Hayes. 2003. Axe-Txe, a broad-spectrum proteic toxin-antitoxin system specified by a multidrug-resistant clinical isolate of *Enterococcus faecium*. *Mol. Microbiol.* 47:1419-1432.
- Hayes, F. 2003. Toxins-antitoxins: plasmid maintenance, programmed cell death, and cell cycle arrest. *Science* 301:1496-1499.
- Hengartner, M. O. 2000. The biochemistry of apoptosis. *Nature* 407:770-776.
- Jaffe, A. T. O., and S. Hiraga. 1985. Effects of the *ccd* function of the F plasmid on bacterial growth. *J. Bacteriol.* 163:841-849.
- Macfadyen, L. P., M. A. Caixia, and R. J. Redfield. 1998. A 3',5' cyclic AMP (cAMP) phosphodiesterase modulates cAMP levels and optimizes competence in *Haemophilus influenzae* Rd. *J. Bacteriol.* 180:4401-4405.
- Madera, M., C. Vogel, S. K. Kummerfeld, C. Chothia, and J. Gough. 2004. The SUPERFAMILY database in 2004: additions and improvements. *Nucleic Acids Res.* 32:235-239.
- Martin, S. J., C. P. Reutelingersperger, A. J. McGahon, J. A. Rader, R. C. Van Schie, D. M. LaFace, and D. R. Green. 1995. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by over expression of Bcl-2 and Abl. *J. Exp. Med.* 182:1545-1556.
- Masuda, Y., K. Miyakawa, Y. Nishimura, and E. Ohtsubo. 1993. *chpA* and *chpB*, *Escherichia coli* chromosomal homologs of the *pem* locus responsible for stable maintenance of plasmid R100. *J. Bacteriol.* 175:6850-6856.
- Megumi, M., H. Hiroshi, L. Zhiquan, I. Kazuhiro, I. Toshi, M. Tadashi, et al. 1999. Occurrence of free D-amino acids and aspartate racemases in hyperthermophilic archaea. *J. Bacteriol.* 181:6560-6563.
- Meier, P., A. Finch, and G. Evan. 2000. Apoptosis in development. *Nature* 407:796-801.
- Metzger, S., I. B. Dror, E. Aizenman, G. Schreiber, M. Toone, J. D. Friesen, M. Cashel, and G. Glaser. 1988. The nucleotide sequence and characterization of the *relA* gene of *Escherichia coli*. *J. Biol. Chem.* 263:15699-15704.
- Morris, S. A., and J. P. Bilezikian. 1983. Evidence that forskolin activates turkey erythrocyte adenylate cyclase through a noncatalytic site. *Arch. Biochem. Biophys.* 220:628-636.
- Naito, Y., T. Naito, and I. Kobayashi. 1998. Selfish restriction modification genes: resistance of a resident R/M plasmid to displacement by an incompatible plasmid mediated by host killing. *Biol. Chem.* 379:429-436.
- Nicholson, D. W., and N. A. Thornberry. 1997. Caspases: killer proteases. *Trends Biochem. Sci.* 22:299-306.
- Patton, T. G., K. C. Rice, M. K. Foster, and K. W. Bayles. 2005. The *Staphylococcus aureus cidC* gene encodes a pyruvate oxidase that effects acetate accumulation and cell death in stationary phase. *Mol. Microbiol.* 56:1664-1674.

32. Raff, M. C. 1992. Social controls on cell survival and cell death. *Nature* **356**:397–400.
33. Rice, K. C., and K. W. Bayles. 2003. Death's toolbox: examining the molecular components of bacterial programmed cell death. *Mol. Microbiol.* **50**: 729–738.
34. Rich, T. C., K. A. Fagan, T. E. Tse, J. Schaack, D. M. Cooper, and J. W. Karpén. 2001. A uniform extracellular stimulus triggers distinct cAMP signals in different compartments of a simple cell. *Proc. Natl. Acad. Sci. USA* **98**:13049–13054.
35. Roberts, R. C., A. R. Ström, and D. R. Helinski. 1994. The *parDE* operon of the broad-host-range plasmid RK2 specifies growth inhibition associated with plasmid loss. *J. Mol. Biol.* **237**:35–51.
36. Ruiz-Echevarria, M. J., A. Berzal-Herranz, K. Gerdes, and R. Diaz-Orejas. 1991. The *kis* and *kid* genes of the *parD* maintenance system of plasmid R1 form an operon that is autoregulated at the level of transcription by the co-ordinated action of the *Kis* and *Kid* proteins. *Mol. Microbiol.* **5**:2685–2693.
37. Schliselfeld, L. H., and M. J. Danon. 1996. Use of fructose-2, 6-diphosphate to assay for phosphofructokinase activity in human muscle. *Clin. Biochem.* **29**:79–83.
38. Seamon, K. B., and J. W. Daly. 1981. Forskolin: a unique diterpene activator of cyclic AMP-generating systems. *J. Cyclic Nucleotide Res.* **7**:201–224.
39. Sharma, A. 1999. *Xanthomonas*, p. 2323–2329. In C. A. Batt, P. Patel, and R. K. Robinson (ed.), *Encyclopedia of food microbiology*. Academic Press, London, England.
40. Sharma, A., P. M. Nair, and S. E. Pawar. 1993. Identification of soyabean strains resistant to *Xanthomonas campestris* pv. *glycines*. *Euphytica* **67**:95–99.
41. Sharma, A., A. N. Syed, and P. M. Nair. 1994. Characterization and plasmid profile of *Xanthomonas campestris* pv. *glycines*. *J. Phytopathol.* **141**:53–58.
42. Sonia, C., P. Ma, L. Cauwenberg, J. Winderickx, M. Crauwels, A. Teunissen, D. Nauwelaers, J. H. de Winde, M.-F. Gorwa, D. Colavizza, and J. M. Thevelein. 1998. Involvement of distinct G-proteins, Gpa2 and Ras, in glucose- and intracellular acidification-induced cAMP signaling in the yeast *Saccharomyces cerevisiae*. *EMBO J.* **17**:3326–3341.
43. Stennicke, H. R., and G. S. Salvesen. 1997. Biochemical characteristics of caspases-3, -6, -7, and -8. *J. Biol. Chem.* **272**:25719–25723.
44. Tompkins, D., and J. Toffaletti. 1982. Enzymatic determination of citrate in serum and urine, with use of the Worthington "ultrafree" device. *Clin. Chem.* **28**:192–195.
45. Tsuchimoto, S., Y. Nishimura, and E. Ohtsubo. 1992. The stable maintenance system *pem* of plasmid R100: degradation of PemI protein may allow PemK protein to inhibit cell growth. *J. Bacteriol.* **174**:4205–4211.
46. Vermes, I., C. Haanen, N. H. Steffens, and C. Reutelingsperger. 1995. A novel assay for apoptosis flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled annexin V. *J. Immunol. Methods* **184**:39–51.
47. White, M. M. 1988. Forskolin alters acetylcholine receptor gating by a mechanism independent of adenylate cyclase activation. *Mol. Pharmacol.* **34**:427–430.
48. Wyllie, A. H. 1993. Apoptosis. *Br. J. Cancer* **67**:205–208.
49. Yarmolinsky, M. B. 1995. Programmed cell death in bacterial populations. *Science* **267**:836–837.