Maximizing Plasmid Stability and Production of Released Proteins in <i>Yersinia enterocolitica</i>

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Virulent serotypes of <i>Yersinia enterocolitica</i> carry a plasmid (pYV) encoding a family of proteins that are released into the medium and whose expression is temperature and calcium regulated. The plasmid is easily lost from cells during their growth in the laboratory. We have used sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting with a monoclonal antibody (3.2C) that is specific for a 25-kDa released protein to show that 32°C is the lowest temperature at which plasmid-encoded proteins are expressed in quantity. The highest calcium concentration allowing full expression of these proteins was 445 to 545 μM at 32°C. Calcium concentrations of 745 μM and above at 37°C completely prevented the loss of pYV during multiple subcultures, while at 32°C, calcium concentrations of 245 μM and greater were sufficient to stabilize the plasmid. Growth of <i>Y. enterocolitica</i> at pH 5.5 was slower than at neutral pH values, but it also resulted in greatly increased stability of pYV. These studies showed that bacterial growth, retention of pYV, and expression of plasmid-encoded proteins may be maximized at 32°C with 445 μM calcium and that pYV stability is enhanced by growth at low pH. These observations suggest new approaches for isolation of plasmid-bearing virulent strains of <i>Y. enterocolitica</i> from samples contaminated with this organism and also may improve our understanding of pYV retention in vivo.

Enteropathogenic strains of <i>Yersinia enterocolitica</i> are recognized as major human and animal pathogens that cause severe diseases, such as gastroenteritis, diarrhea, and mesenteric lymphadenitis (7, 8). Although the virulence factors of <i>Yersinia</i> are complex, a 70- to 76-kb plasmid (pYV) is specifically required for pathogenicity (7, 8). The pYV plasmid encodes a number of important virulence and virulence-associated proteins that are synthesized and released into the medium. Because these proteins originally were found in the outer membrane fraction of bacterial extracts (6), they are by convention called Yops (<i>Yersinia</i> outer membrane proteins). However, it has been demonstrated that they are actually secreted proteins and that localization in the outer membrane is transient (13). The Yop genes are coordinately regulated by a virulence regulon called the low-calcium response (Lcr) regulon (16). Most of these proteins are only expressed efficiently in vitro when the bacteria are cultured at 37°C and under calcium-restricted conditions (10). Li et al. (12) used a monoclonal antibody (3.2C) and Western blotting as a very sensitive means of detecting expression of one of the major released proteins, the 25-kDa protein YopE. Synthesis of YopE was sharply restricted in <i>Y. enterocolitica</i> O:8 following a temperature shift from 26°C to 37°C and at all calcium concentrations above 345 μM (12).

There are some common problems related to the study and isolation of pathogenic plasmid-bearing <i>Y. enterocolitica</i> (YEP') strains from natural sources or clinical samples. Cold enrichment procedures are used, and this necessitates long incubation times, often exceeding several weeks. Shorter incubations conducted at higher temperatures have the disadvantage of overgrowth by other organisms and rapid loss of pYV plasmid (7). Bhaduri et al. (2) refined the selective enrichment procedure to reduce the total time required to 120 h for the isolation of pathogenic YEP' strains. Since expression of Yops is dependent on temperature, calcium, and pH, the objects of the present study were to investigate the roles of these variables in expression of the 25-kDa protein YopE and other Yops produced by <i>Y. enterocolitica</i> and to establish optimal growth conditions that minimize loss of plasmid. These parameters might be used to speed detection and isolation of the organisms from clinical, food, and environmental samples as well as provide explanations of how the pYV plasmid is retained under both environmental and in vivo conditions.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. YEP’ serotype O:3 (CI) was obtained from the Centers for Disease Control and Prevention, Atlanta, Ga., and serotype O:8 (WA) was obtained from stock cultures of the Eastern Regional Research Center, USDA Agricultural Research Service, Wyndmoor, Pa. Stock cultures were stored as cell suspensions at −70°C in 50% glycerol, and their authenticity was confirmed periodically by PCR techniques and tests for plasmid retention. Brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.), calcium-adequate (1,500 μM) BHI agar (BHA) (Difco), and low-calcium (238 μM) Congo red (CR) (Sigma Chemical Co., St. Louis, Mo.)-BHI agarose (CR-BHO) (Gibco BRL, Gaithersburg, Md.) were prepared as described previously (1, 5). The avirulent plasmidless derivatives (YEP') were obtained from large, flat colonies which emerged spontaneously from YEP’ culture growing at 37°C on CR-BHO (5). Cells were cultivated in BHI broth at 26°C with shaking overnight unless otherwise indicated (2).

Growth experiments. YEP’ and YEP’ strains were precultured in BHI broth at 26°C with shaking overnight. The optical density of the preculture was adjusted to 0.4 at 600 nm, and the preculture was diluted 1:20 with fresh BHI broth. Two sets of cultures were prepared. Filler-sterilized calcium chloride solution (Sigma Chemical Company) was added to the BHI broth in one set to give the desired final concentration of 345 μM (245 μM in BHI broth plus 100 μM calcium).
calcium chloride supplement) (12). The temperature was shifted from 26°C to 28, 30, 32, 35, or 37°C, and incubation continued with shaking for up to 6 h. The other set was supplemented with various levels of calcium to a final concentration of 245, 345, 745, or 1,245 μM. The temperature was shifted from 30°C to 32°C, and incubation continued with shaking for up to 6 h. Rates of growth were determined by linear regression analysis of the logarithm of the A600 from 0 to 4 h of growth. r² values were >0.97.

Isolation of Yops, SDS-PAGE, and Western blotting. Yops were precipitated from the culture supernatant with 40% ammonium sulfate and dissolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer (10, 12). Electrophoresis with 12% polyacrylamide separation gels and Coomassie brilliant blue staining were carried out as previously described (11). Following electrophoresis, gels were washed in distilled water and transfer buffer (192 mM glycine, 25 mM Tris, 20% methanol [pH 8.0]). Proteins were transferred electrically to polyvinylidene difluoride membranes (pore size of 0.45 μm; Millipore, Bedford, Mass.) with a Trans-Blot transfer cell (Bio-Rad Laboratories, Hercules, Calif.) and a constant voltage of 30 V at 4°C overnight. After protein transfer, the polyvinylidene difluoride membrane was washed with phosphate-buffered saline (PBS) containing 0.2% (vol/vol) Tween 20 (PBST) and blocked in blocking solution (PBST containing 5% nonfat milk) at room temperature for 10 min. The membrane then was incubated for 1 h at room temperature with primary monoclonal antibody 3.2C (12), which was diluted 1:5,000 in blocking solution. After being washed with PBST, the membrane was blocked again in blocking solution for 10 min at room temperature. The secondary antibody of goat anti-mouse immunoglobulin G (heavy plus light chains) coupled to horseradish peroxidase (Boehringer Mannheim, Indianapolis, Ind.) diluted 1:20,000 with blocking solution was added. After incubation at room temperature for 20 min, the membrane was washed with PBST, and the ECL (enhanced chemiluminescence) reagent (DuPont, Boston, Mass.) was added. The blot was incubated for 2 min and exposed to X-ray film (DuPont, Wilmington, Del.) for 2 min, and then the film was developed.

The effect of pH on growth. Y. enterocolitica O:3 (CI) YEP⁺ cells were grown in BHI broth at 26°C overnight with shaking. The optical density of the culture was adjusted to 0.4 at 600 nm. The bacterial culture was diluted 1:20 with fresh BHI broth adjusted to various pH values without (final concentration, 245 μM) or with a supplement of 200 μM calcium chloride (final concentration, 445 μM). The original pH of the BHI broth was 7.2. The pH was adjusted to 4.5, 5.5, 6.5, 7.0, 7.5, 8.0, or 8.5 with HCl or NaOH (4). The temperature was then shifted from 26°C to 37°C, and incubation continued with shaking overnight.

Results and Discussion

The pYV plasmid in YEP⁺ strains appears to be stably retained in vivo at 37°C and also at lower temperatures in the environment. However, the plasmid is very unstable at 37°C in vitro under the culture conditions often used for bacterial isolations. First, we first investigated whether Yops were produced in culture at any temperatures below 37°C. Y. enterocolitica serotype O:3 (CI) was grown in BHI broth with 100 μM calcium chloride supplement (final calcium concentration, 345 μM) (12) at various temperatures from an inoculum grown at 26°C. As shown in Fig. 1a, release of protein was observed at 37, 35, and 32°C and indicated that the cutoff temperature for expression of Yops by the YEP⁺ O:3 strain is about 32°C. Production of YopE was confirmed by Western blotting with monoclonal antibody 3.2C (12) (Fig. 1c). Similar results were observed for a clinical isolate of Y. enterocolitica O:8 (WA) (Fig. 1b). Released proteins never were detected from any cells that had lost the pYV plasmid (YEP⁻ cells).

Other studies (9, 10) have shown that expression of released proteins encoded by the plasmid was strongly enhanced when the organism was grown in media containing chelators, such as EGTA. Presumably EGTA forms complexes with calcium ions and makes them unavailable. Under these growth conditions, significant amounts of Yops were produced at 37°C, but these were not observed for cultures in exponential- or stationary-phase growth at 20 or 30°C. Michiels et al. (13) reported that several Yersinia outer membrane proteins were released into culture supernatants under calcium-restricted conditions. A 25-kDa protein (YopE) was found to be one of the major released proteins. Recently, Li et al. (12) detected large amounts of 25-kDa YopE protein in cultures at the late exponential growth phase when Yersinia was grown at 37°C in a broth medium such as BHI broth without the need for depletion of calcium by the use of calcium chelators.

We next evaluated calcium regulation of Yop expression at 32°C. YEP⁺ serotype O:3 (CI) cells were grown in BHI broth supplemented with the different concentrations of calcium chloride. Expression of released proteins occurred at the lower calcium concentrations used (245 to 545 μM) at the cutoff temperature of 32°C. Expression of YopE and other released proteins decreased sharply at calcium concentrations above
545 μM (Fig. 2a and b). This indicated that the calcium concentration present in BHI broth (245 μM) is sufficient for optimum expression of released proteins of YEP strains at either 32 or 37°C and that significant expression of Yops occurred at even greater calcium concentrations.

The possibility that the level of calcium in the medium might influence plasmid stability was investigated after it was observed in our earlier experiments that higher percentages of YEP colonies were found after growth at elevated calcium levels. The YEP O:3 (CI) strain was subcultured every 24 h for up to 9 days at 37 and 32°C in BHI broth supplemented with different concentrations of calcium chloride. The bacterial cells were plated, and colonies were screened for the presence of plasmid by crystal violet binding (1), CR uptake (5), and PCR amplification assays (3). When YEP cells were grown at 37°C, they totally lost the plasmid after overnight culture in BHI broth without supplemental calcium (Fig. 3a). Some clumping of cells was observed as cells altered their phenotype at 37°C. A dramatic improvement in plasmid retention was observed at calcium concentrations of 745 and 1,245 μM, while the organism started to lose plasmid at day 4 at 445 μM. Culture at 32°C showed that complete plasmid stability was obtained at a lower calcium concentration than at 37°C (Fig. 3b). Only a slight loss of pYV occurred after 9 days of subculture at 32°C in BHI broth without the calcium supplement, and the plasmid was retained completely in all of the supplemented cultures. Thus, these results showed that a temperature of 32°C and calcium concentrations above 245 μM completely prevented loss of the pYV plasmid while permitting production of released proteins.

The growth rates for Y. enterocolitica were determined during each of the temperature and calcium concentration experiments. As shown in Fig. 4, the rate of growth of YEP cells was lower than that of YEP cells at each temperature examined, and inhibition in the growth rate increased with increasing temperature. These results suggest that YEP cells grown at temperatures above 30°C are under stress, possibly due to increased synthesis of Yops. The various calcium concentrations did not markedly affect the growth of YEP strains at 32°C (data not shown). These results were to be expected, since even the lowest calcium concentration used (245 μM in BHI broth) was above the level reported to cause low-calcium growth restriction (12, 16).

Finally, we examined the effects of pH on growth and plasmid stability in the YEP strain by culturing the organism at 37°C in BHI broth with or without calcium chloride supplementation. The organism grew best at pH 6.5 to 8.0, and there was no pronounced difference between the extent of growth at 245 μM calcium and that at 445 μM calcium at any of the pH
values tested (Fig. 5a). The virulence plasmid was retained in the presence of 445 μM calcium chloride at all of the pH values tested (Fig. 5b). However, when the bacteria were grown over-

night in BHI broth without supplemental calcium chloride (245 μM), they completely lost the plasmid at pH 7.2 (Fig. 3a and 5b) but retained about 20% YEP+ cells at pH 8.0, 50% YEP+ cells at pH 6.5, and 100% YEP+ cells at pH 5.5 and 4.5 (Fig. 5b). Bacterial growth was depressed at 37°C and low pH, but loss of pYV plasmid was prevented completely, even at low calcium concentrations.

The current results provide new approaches for improving enrichment, isolation, and identification of pathogenic YEP strains of Y. enterocolitica from food, clinical, and environmental sources while maximizing pYV retention by calcium and pH adjustment. Experiments are under way to combine enrichment at higher temperatures, as proposed by Bhaduri et al. (2), with these new observations. Our results also may offer insight into how the plasmid of Yersinia sp. is stabilized in vivo (16). The high concentration of calcium of approximately 1 mM in body fluids would prevent plasmid loss even at 37°C. Recent evidence has demonstrated how Yops might be locally produced under these high calcium conditions. Pettersson et al. (15) showed that when Yersinia pseudotuberculosis comes in contact with eukaryotic cells, the microbe-host interaction (14) leads to Yop synthesis and direct translocation to recipient cells via the Yop type III secretion system. Derepression of yop genes was necessarily preceded by secretion of a negative regulator of Yop expression. The in vitro low calcium response leading to Yop secretion then may be viewed as a result of an artificial opening of the export channels of the type III secretion system. In necrotic lesions, in which the pH might be below pH 5, the virulence plasmid would be stable regardless of the calcium level. Bacteria taken up by macrophages or other cells might find themselves in a low-pH, low-calcium environment in which Yops could be expressed. The plasmid should be completely stable under those conditions.

REFERENCES