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A Plasmid-Encoded Surface Protein Found in Chronic-Disease Isolates of *Coxiella burnetii*

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The *cbbE'* gene codes for the E' protein of *Coxiella burnetii* and was detected in genomic DNA from all known human isolates of the biotzere strain but not in DNA from the other five strains of *C. burnetii*. The biotzere strain is strictly associated with chronic disease in humans. Extrinsic iodination of biotzere strain cells radiolabeled a 55-kDa protein which comigrated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis with the E' protein synthesized in vitro from recombinants containing the *cbbE'* gene. The ^{125}I-labeled 55-kDa protein was immunoprecipitated with polyclonal anti-E' antiserum, confirming its identity as E'. Predicted secondary structure of the E' polypeptide shows six regions of beta-sheet structure and an alpha-helix near the C terminus with adequate lengths to span a membrane. The predicted hydropathy profile of E' is similar to profiles of known outer membrane proteins and corroborates the biochemical data, indicating that the protein is located in the outer membrane of *C. burnetii*.

Coxiella burnetii is an obligate intracellular bacterium which causes Q fever in humans. The disease usually presents as an acute flulike illness that is self-resolving or is effectively treated with tetracycline (6). In 1 to 10% of cases, infection is chronic in nature and can result in endocarditis (13). Chronic cases of Q fever respond poorly to conventional treatments and usually require prolonged therapy with antibiotics such as doxycycline in combination with rifampin. Early combination therapy with quinolone antibiotics also holds promise for treatment of chronic forms of the disease (35, 36).

The obligate intracellular parasitism of *C. burnetii* makes a number of analyses difficult, and molecular aspects of its pathogenesis are poorly defined. Chronic infection was once thought to develop in individuals who were immunocompromised or who had a history of heart valve damage. However, recent evidence shows that certain strains of *C. burnetii* are uniquely associated with chronic or acute cases of the disease (16). A strain designation based on genomic restriction fragment length polymorphisms and plasmid content has been proposed, separating *C. burnetii* isolates into six strains or genomic groups (11, 16). Biochemical differences between strains have also been reported for lipopolysaccharides and surface proteins (9, 22). Only isolates from the biotzere strain (group IV) and the corazon strain (group V) have been isolated from chronic cases of the disease. Hamilton (group I), vacca (group II), and rasche (group III) strains are associated with cases of acute Q fever, while the dod strain (group VI) has not yet been isolated from humans (16). Prescribing an appropriate antibiotic therapy for Q fever would be facilitated by initially determining the acute or chronic disease potential of a particular isolate. However, there is currently no rapid clinical means of distinguishing among *C. burnetii* strains associated with the chronic and acute forms of the disease.

There is a correlation between plasmid sequences and the virulence potential of *C. burnetii*. The QpH1 plasmid of *C. burnetii* is found in isolates from cases of acute Q fever (genomic groups I, II, and III), while the QpRS plasmid and chromosomally integrated sequences with homology to QpRS (30) are found in chronic endocarditis isolates from genomic groups IV and V, respectively (16, 28, 32). DNA sequences which are unique to each plasmid type are being characterized to ascertain whether virulence determinants which are involved in the acute or chronic nature of the disease are present. Unique sequences and their respective gene products also represent potential targets for diagnosing a particular isolate of *C. burnetii* as belonging to a strain which causes acute or chronic disease (17).

A QpRS-specific gene termed *cbbE'* was recently cloned, sequenced, and expressed in *Escherichia coli* (21). In this report we present data showing that the E' protein coded by *cbbE'* is surface exposed on the bacterium and that the gene is unique to a strain of *C. burnetii* which causes chronic disease in humans.

C. burnetii was grown and its plasmid and genomic DNAs were purified as previously described (27) from the group I isolate Nine Mile RSA 493; group II isolate M-44 RSA459; group III isolate Idaho goat Q195; group IV isolates priscilla Q177, Canada goat Q218, K Q154, P Q173, F Q228, and H WSU101; group V isolate Ko Q229; and group VI isolate Dugway 7E9-12. The priscilla Q177 isolate was used as a representative of the biotzere strain (genomic group IV).

Specificity of the *cbbE'* plasmid gene to genomic group IV was shown by DNA-DNA hybridization (Fig. 1). An earlier report showed that the gene was unique to the QpRS plasmid (21). DNA probe preparation and hybridization procedures have been described previously (21). At a stringency of approximately 7% DNA mismatch, the *cbbE'* gene was not detected in the genomic DNAs of isolates from any genomic groups associated with acute disease, i.e., genomic groups I, II, and III, nor was it detected in DNA from genomic groups V and VI. Only group IV genomic DNA from human endocarditis isolates K, P, F, and H plus the genomic DNA from goat abortion isolates priscilla and Canada goat was found to contain an *Eco*RI fragment of 3.6 kb with homology to *cbbE'* (Fig. 1). A 3.6-kb *Eco*RI fragment was previously

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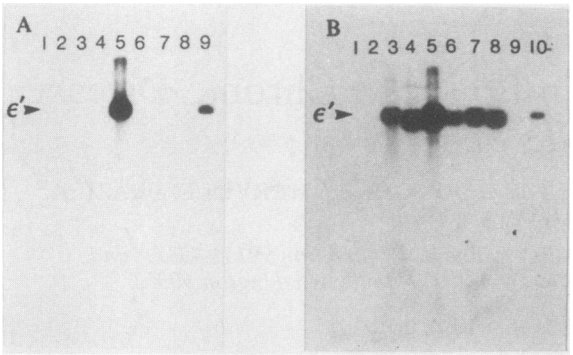

FIG. 1. Detection of the *cbbE'* gene of *C. burnetii* by DNA-DNA hybridization. Genomic DNAs from the six strains of *C. burnetii* (A) and from six isolates belonging to the biotzere strain (genomic group IV) (B) were digested with *Eco*RI, electrophoresed in 1% agarose, and then blotted and probed with the internal *Pst*I fragment of *cbbE'* (21). (A) Lanes: 1, lambda *Hin*dIII DNA; 2, Hamilton strain (group I) (RSA 493); 3, vacca strain (group II) (M-44 RSA459); 4, rasche strain (group III) (Q195); 5, biotzere strain (group IV) (Q177); 6, corazon strain (group V) (Q229); 7, dod strain (group VI) (7E9-12); 8, empty; 9, pQME1. (B) Lanes: 1, lambda *Hin*dIII DNA; 2, Nine mile (RSA 493); 3, priscilla (Q177); 4, Canada goat Q218; 5, K (Q154); 6, P (Q173); 7, F (Q228); 8, H (WSU101); 9, empty; 10, pQME1.

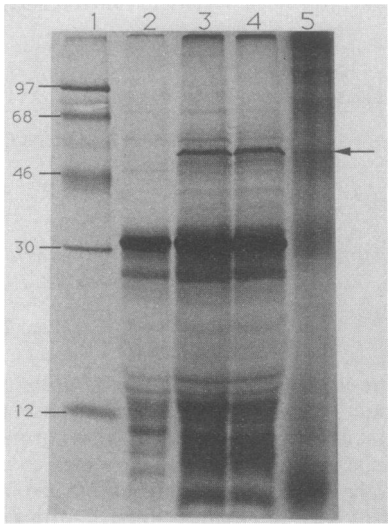

FIG. 2. Detection of the E' protein by extrinsic radioiodination of *C. burnetii* cells and comigration of the iodinated protein with E' protein synthesized by IVTT. Lanes: 1, ^{14}C-labelled M_r standards; 2, IVTT of pUC19; 3, IVTT of pQME1; 4, IVTT of pQME2; 5, surface-iodinated cells of the priscilla isolate. The position of E' is indicated by an arrow. Molecular masses (kilodaltons) are on the left.

cloned from the QpRS plasmid and contains the entire *cbbE'* gene (20, 21). The 3.6-kb *Eco*RI band from the *cbbE'*-containing recombinant pQME1 (Fig. 1A, lane 9, and B, lane 10) comigrates with the genomic DNA hybridization signals on the Southern blots. Since each of the group IV isolates (Fig. 1B) is known to possess the QpRS plasmid (16), the hybridization data suggest that *cbbE'* is confined to the same *Eco*RI fragment of the plasmid.

The surface localization of E' was first hypothesized when polyclonal antiserum generated against outer membrane material from the priscilla isolate recognized the E' protein in immunoblots containing cell lysates of *E. coli* expressing the *cbbE'* gene (21). Surface-exposed proteins of *C. burnetii* are both numerous and immunogenic (34), and approximately 35 proteins can be labeled by extrinsic radioiodination of the rickettsiae (33). Interstrain differences in surface iodination profiles suggest that the array of outer membrane proteins may differ between strains of *C. burnetii* (22). To determine whether E' is surface exposed, approximately 0.1 mg (wet weight) of freshly isolated *C. burnetii* cells (priscilla isolate) was radioiodinated by the methods of Markwell (18). Intact cells were washed and resuspended in 0.5 ml of phosphate-buffered saline (pH 7.4; 4°C) and then equilibrated for 5 min at 25°C with five Iodobeads (Pierce Chemical, Rockford, Ill.). Na^{125}I was then added to a final concentration of 1 mCi/ml. The reaction proceeded for 15 min at 25°C with agitation at 5-min intervals and was stopped by removing the beads. Samples were diluted 1:1 (vol/vol) in Laemmli sample buffer (15) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or stored at −20°C. In vitro transcription and translation (IVTT) was done on recombinant plasmids pQME1 and pQME2 as described previously (20, 21) to analyze E' protein synthesized in vitro.

Radioiodination, radioimmunoprecipitation analysis (RIPA), and IVTT samples were analyzed by SDS-PAGE (12.5% acrylamide [wt/vol]), by the methods of Laemmli (15). All samples were boiled for 5 min in Laemmli sample buffer, and the gels were run at 7.5 mA to increase resolution of the protein bands. Autoradiographs were produced by exposing dried gels to X-ray film for 2 to 5 h at 25°C.

The group IV priscilla isolate of *C. burnetii* showed a prominently iodinated protein species of 55 kDa (Fig. 2, lane 5) which comigrated during SDS-PAGE with E' protein synthesized in vitro (Fig. 2, lanes 3 and 4). To determine whether the surface-labeled 55-kDa protein was the E' protein, anti-E' antiserum was generated by using two synthetic peptides from the predicted amino acid sequence (21). Peptides from the N- and C-terminus regions of the polypeptide were chosen on the basis of predicted antigenicity (12) and a high probability of surface exposure (5) with the Pustell computer package (International Biotechnologies, New Haven, Conn.). The E' peptides consisted of KEQELFEIRE (amino acid residues 71 to 80) and KTQRL GTGNT (residues 427 to 436) and were coupled to keyhole limpet hemocyanin by the glutaraldehyde method (2). A 750-μg portion of each peptide-carrier conjugate was combined in 1 ml of physiological saline (0.15 M) and used to generate rabbit anti-E' polyclonal antiserum as previously described (21).

RIPA of surface-iodinated *C. burnetii* was performed by a modification of the methods of Postle and Skare (25). Briefly, 25 μl of the iodinated cells in Laemmli sample buffer (as described above) was diluted with 1 ml of RIPA buffer (25) and microcentrifuged for 10 min. Twenty microliters of rabbit preimmune or anti-E' serum was added to the supernatant, mixed, and incubated on ice for 60 min. Sepharose-protein A (120 μl of a 25% solution [wt/vol]) was added, and the mixture was incubated for 30 min on ice with gentle vortexing every 5 min. Beads were washed three times with 0.5 ml of RIPA buffer followed by a 15-s Microfuge spin and then washed a final time in 0.5 ml of 0.05 M NaCl–0.01 M Tris-HCl (pH 7.4)–1 mM EDTA (NTE). The NTE buffer was decanted, and the beads were resuspended in 25 μl of Laemmli sample buffer for analysis by SDS-PAGE. RIPA

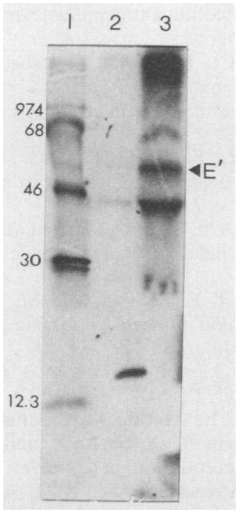

FIG. 3. RIPA of the E′ protein from surface-iodinated cells of the priscilla isolate of *C. burnetii* by the general methods of Postle and Skare (25). Lanes: 1, ^{14}C-labelled M_r standards (kilodaltons); 2, RIPA with preimmune rabbit serum; 3, RIPA with anti-E′ antiserum. The position of E′ is indicated by an arrow.

with anti-E′ antiserum precipitated the ^{125}I-labeled 55-kDa protein, confirming its identity as E′ (Fig. 3, lane 3). Labeled proteins of 28, 45, and 65 kDa were coprecipitated from the priscilla isolate. Preimmune rabbit serum precipitated a 14-kDa protein and weakly precipitated the 45-kDa protein (Fig. 3, lane 2). It is possible that the coprecipitated polypeptides are antigenically cross-reactive with E′ or alternatively that they coprecipitated as a protein and/or lipopolysaccharide complex.

RIPA data (Fig. 3) were corroborated by indirect immunofluorescence with anti-E′ antiserum. Intact cells of the priscilla isolate fluoresced strongly and more intensely than cells treated in a similar fashion with preimmune rabbit serum. In keeping with the DNA hybridization data, which show that the gene coding for the E′ protein is unique to the biotzere strain (Fig. 1), immunofluorescence of intact cells of the Hamilton strain (group I) treated with anti-E′ serum was consistently less than immunofluorescence observed with cells from the biotzere strain (data not shown).

Because outer membrane proteins lack hydrophobic stretches greater than 20 amino acids and have similar overall hydropathies (23), a comparison was made between hydropathy profiles of the E′ protein with the *E. coli* cytoplasmic membrane protein TetA (24) and the *E. coli* outer membrane protein TolC (8) (Fig. 4). The predicted amino acid sequence of the E′ protein (21) was computer analyzed for hydropathy (14) and secondary structure prediction (3) with the University of Wisconsin Genetics Computer Group software (4). Mean areas under the hydrophobic peaks from the plots in Fig. 4 were obtained by using a digitizer plus the Sigma Scan software (Jandel Scientific, Corte Madera, Calif.) and were calculated as 16.8 for E′, 14.0 for TolC and 73.5 for TetA. These data suggest that the hydropathy of E′ is more similar to that of TolC and that both E′ and TolC are predominantly hydrophilic. Hydrophobic stretches of E′ range from 6 to 20 amino acid residues, with a mean value of 9.4 residues, comparing favorably with the mean hydrophobic stretch length of 9.7 residues in TolC. In contrast, the plasma membrane protein TetA shows a more hydrophobic protein,

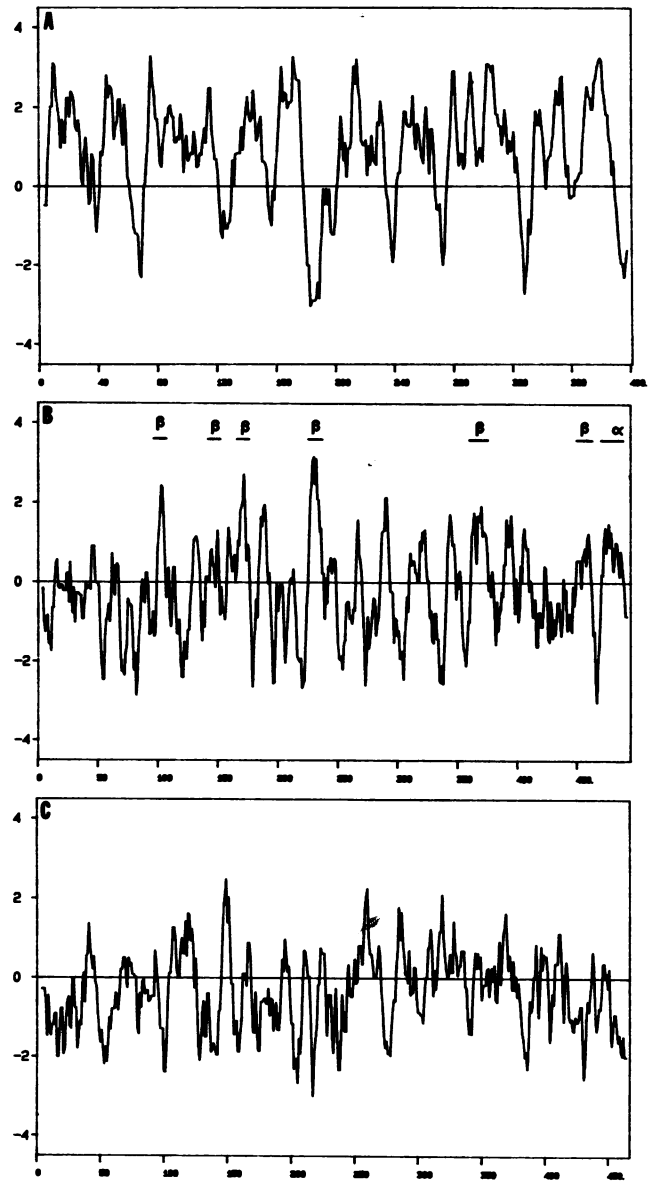

FIG. 4. Comparison of Kyte and Doolittle (14) hydropathy plots of *E. coli* TetA (A) (24), *C. burnetii* E′ (B) (21), and *E. coli* TolC (C) (8) proteins. Values on the *y* axes represent hydrophobicity, where positive values represent predicted hydrophobic regions. Values of the *x* axes represent residues in the amino acid sequence. Hydrophobic peaks of E′ with adequate lengths to span a membrane are overlined, and the predicted structural motif is indicated by an alpha or beta (B).

with a mean hydrophobic peak area of 73.5 and a mean hydrophobic stretch length of 27 residues.

Approximately 74% of the hydrophobic segments of E′ are predicted as beta-sheet structure (3). Six potential membrane-spanning domains of E′ with predicted beta-sheet structures and with adequate lengths to span a membrane are indicated in Fig. 4. The most hydrophobic beta-sheet structure of E′ is 12 amino acids and is located near the middle of the sequence (residues 224 to 235). A hydrophobic region of 20 amino acids occurs near the C terminus of E′ and is predicted as an alpha-helix (Fig. 4). This region is also of

adequate length to span a membrane (1) and together with beta-sheet structures may serve to anchor the E′ protein.

Radioiodination, RIPA, and hydropathy data all suggest that E′ is surface exposed on genomic group IV *C. burnetii* (biotzere strain). Both the *cbbE′* gene and the E′ protein represent two potential targets for identifying a specific strain of *C. burnetii* which is associated with chronic endocarditis in humans. Analysis of other strain-specific genes or proteins of *C. burnetii* could provide a basis for distinguishing among all isolates of this bacterium. The ability to identify an isolate as having the potential to cause chronic or acute disease will facilitate the prescribing of a proper antibiotic regimen.

Although the function of the E′ protein is unknown, its surface exposure and coding on a plasmid harbored by a chronic strain of *C. burnetii* suggest a possible role in virulence. Plasmid genes are known virulence determinants in a number of bacteria, including enteroinvasive *E. coli* (31), *Shigella* spp. (19, 26, 29), and *Salmonella* spp. (7, 10). Continued efforts in this area will help to clarify the pathogenesis of this bacterium.

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REFERENCES

1. **Adams, G. A., and J. K. Rose.** 1985. Structural requirements of a membrane-spanning domain for protein anchoring and cell surface transport. Cell **41**:1007–1015.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. F. Seidman, J. A. Smith, and K. Struhl.** 1988. Current protocols in molecular biology, p. 11.15.2. Wiley and Sons, New York.
3. **Chou, P. Y., and G. D. Fasman.** 1978. Prediction of secondary structure of proteins from their amino acid sequence. J. Mol. Biol. **120**:45–120.
4. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12**:387–395.
5. **Emini, E. A., J. V. Hughes, D. S. Perlow, and J. Boger.** 1985. Induction of hepatitis- A virus-neutralizing antibody by a virus-specific synthetic peptide. J. Virol. **55**:836–839.
6. **Gilman, A. G., L. S. Goodman, and A. Gilman.** 1980. The pharmacological basis of therapeutics, p. 1092. Macmillan, New York.
7. **Gulig, P. A., and R. Curtiss III.** 1987. Plasmid-associated virulence of *Salmonella typhimurium*. Infect. Immun. **55**:2891–2901.
8. **Hackett J., R. Misra, and P. Reeves.** 1983. The TolC protein of *Escherichia coli* K12 is synthesized in a precursor form. FEBS Lett. **156**:307–310.
9. **Hackstadt, T.** 1986. Antigenic variation in the phase I lipopolysaccharide of *Coxiella burnetii* isolates. Infect. Immun. **52**:337–340.
10. **Heffernan, E. J., J. Fierer, G. Chikami, and D. Guiney.** 1987. Natural history of oral *Salmonella dublin* infection in BALB/c mice: effect of an 80-kilobase-pair plasmid on virulence. J. Infect. Dis. **155**:1254–1259.
11. **Hendrix, L., J. E. Samuel, and L. P. Mallavia.** 1991. Differentiation of *Coxiella burnetii* isolates by restriction endonuclease-digested DNA separated on SDS-PAGE. J. Gen. Microbiol. **137**:269–276.
12. **Jameson, B. A., and H. Wolf.** 1988. The antigenic index: a novel algorithm for predicting antigenic determinants. Comput. Appl. Biosci. **4**:181–186.
13. **Kimbrough, R. C., III, R. A. Ormsbee, M. Peacock, W. R. Rogers, R. W. Bennetts, J. Roof, A. Krause, and C. Gardner.** 1979. Q fever endocarditis in the United States. Ann. Intern. Med. **91**:400–402.
14. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157**:105–132.
15. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
16. **Mallavia, L. P., J. E. Samuel, and M. E. Frazier.** 1991. The genetics of *Coxiella burnetii*; etiologic agent of Q fever and chronic endocarditis, p. 259–284. *In* J. C. Williams and H. Thompson (ed.), Q fever: the biology of *Coxiella burnetii*. CRC Press, Boca Raton, Fla.
17. **Mallavia, L. P., L. L. Whiting, M. F. Minnick, R. A. Heinzen, D. Reschke, M. Foreman, O. G. Baca, and M. E. Frazier.** 1990. Strategy for differentiation of *Coxiella burnetii* strains using polymerase chain reaction. Ann. N. Y. Acad. Sci. **590**:572–581.
18. **Markwell, M. A. K.** 1982. A new solid-state reagent to iodinate proteins. I. Conditions for the efficient labeling of antiserum. Anal. Biochem. **125**:427–432.
19. **Maurelli, A. T., B. Baudry, H. d'Hauteville, T. L. Hale, and P. J. Sansonetti.** 1985. Cloning of plasmid DNA sequences involved in invasion of HeLa cells by *Shigella flexneri*. Infect. Immun. **49**:164–171.
20. **Minnick, M. F., R. A. Heinzen, R. Douthardt, L. P. Mallavia, and M. E. Frazier.** 1990. Analysis of QpRS-specific sequences from *Coxiella burnetii*. Ann. N. Y. Acad. Sci. **590**:514–522.
21. **Minnick, M. F., R. A. Heinzen, M. E. Frazier, and L. P. Mallavia.** 1990. Characterization and expression of the *cbbE′* gene of *Coxiella burnetii*. J. Gen. Microbiol. **136**:1099–1107.
22. **Moos, A., and T. Hackstadt.** 1987. Comparative virulence of intra- and interstrain lipopolysaccharide variants of *Coxiella burnetii* in the guinea pig model. Infect. Immun. **55**:1144–1150.
23. **Nau, C. D., and J. Koninsky.** 1989. Evolutionary relationship between the TonB-dependent outer membrane transport proteins: nucleotide and amino acid sequences of the *Escherichia coli* colicin I receptor gene. J. Bacteriol. **171**:1041–1047.
24. **Nguyen, T. T., K. Postle, and K. Bertrand.** 1983. Sequence homology between the tetracycline resistance determinants of TN10 and pBR322. Gene **25**:83–92.
25. **Postle, K., and J. T. Skare.** 1988. *Escherichia coli* TonB protein is exported from the cytoplasm without proteolytic cleavage of its amino terminus. J. Biol. Chem. **263**:11000–11007.
26. **Sakai, T., C. Sasakawa, S. Makino, K. Kamata, and M. Yoshikawa.** 1986. Molecular cloning of a genetic determinant for Congo red binding ability which is essential for the virulence of *Shigella flexneri*. Infect. Immun. **51**:476–482.
27. **Samuel, J. E., M. E. Frazier, M. L. Kahn, L. S. Thomashow, and L. P. Mallavia.** 1983. Isolation and characterization of a plasmid from phase I *Coxiella burnetii*. Infect. Immun. **41**:488–493.
28. **Samuel, J. E., M. E. Frazier, and L. P. Mallavia.** 1985. Correlation of plasmid type and disease caused by *Coxiella burnetii*. Infect. Immun. **49**:775–779.
29. **Sasakawa, C., K. Kamata, T. Sakai, S. Y. Murayama, S. Makino, and M. Yoshikawa.** 1986. Molecular alteration of the 140-megadalton plasmid associated with loss of virulence and Congo red binding activity in *Shigella flexneri*. Infect. Immun. **51**:470–475.
30. **Savinelli, E. A., and L. P. Mallavia.** 1990. Comparison of *Coxiella burnetii* plasmids to homologous chromosomal sequences present in a plasmidless endocarditis-causing isolate. Ann. N. Y. Acad. Sci. **590**:523–533.
31. **Small, P. L. C., and S. Falkow.** 1988. Identification of regions on a 230-kilobase plasmid from enteroinvasive *Escherichia coli* that are required for entry into HEp-2 cells. Infect. Immun. **56**:225–229.
32. **Vodkin, M. H., J. C. Williams, and E. H. Stephenson.** 1986. Genetic heterogeneity among isolates of *Coxiella burnetii*. J. Gen. Microbiol. **132**:455–463.

33. **Williams, J. C.** 1981. Iodination of *Coxiella burnetii* cells: optimal conditions for the iodination of exposed surface proteins, p. 473–482. *In* W. Burgdorfer and R. L. Anacker (ed.), Rickettsiae and rickettsial diseases. Academic Press, New York.
34. **Williams, J. C., and S. Stewart.** 1984. Identification of immunogenic proteins of *Coxiella burnetii* phase variants, p. 257–262. *In* L. Leive and D. Schlessinger (ed.), Microbiology—1984. American Society for Microbiology, Washington, D.C.
35. **Yeaman, M. R., L. A. Mitscher, and O. G. Baca.** 1987. In vitro susceptibility of *Coxiella burnetii* to antibiotics, including several quinolones. Antimicrob. Agents Chemother. **31**:1079–1084.
36. **Yeaman, M. R., M. J. Roman, and O. G. Baca.** 1989. Antibiotic susceptibilities of two *Coxiella burnetii* isolates implicated in distinct clinical syndromes. Antimicrob. Agents Chemother. **33**:1052–1057.