genes of other bacterial species (14). Mutations within this locus yield chlorine-sensitive, smooth colony variants that can not form biofilm. *vps* genes are clustered in two regions in the *V. cholerae* chromosome (14). One cluster harbors genes *vpsA* through *vpsK*, and the other one harbors genes *vpsL* through *vpsQ*. Rugose variants lacking *vpsA* or *vpsL* do not produce EPS and exhibit a smooth colonial morphology (14). Several invesigators have shown the existence of two positive regulators of *vps* genes, VpsR (57) and VpsT (58), and two negative regulators, HapR (60, 61) and CytR (62). Yildiz et al. (63) identified 124 differentially regulated genes by microarray expression profiling studies of the rugose and smooth variants of the same strain. Bioinformatics analysis of these expression data shows that 'rugosity' and 'smoothness' are determined by a complex hierarchy of positive and negative regulators, which also affect the biofilm, surface hydrophobicity and motility phenotypes of *V. cholerae* (63).

An immunoelectron microscopic examination demonstrated that there is an epitope common to the exopolysaccharide antigen of *V. cholerae* O1 and that of O139 (13). The antiserum against *V. cholerae* O1 EPS (12) was reactive only with *V. cholerae* O139 rugose variant and not with smooth variant (Fig. 6). The gold particles were specifically bound to the slime layer surrounding

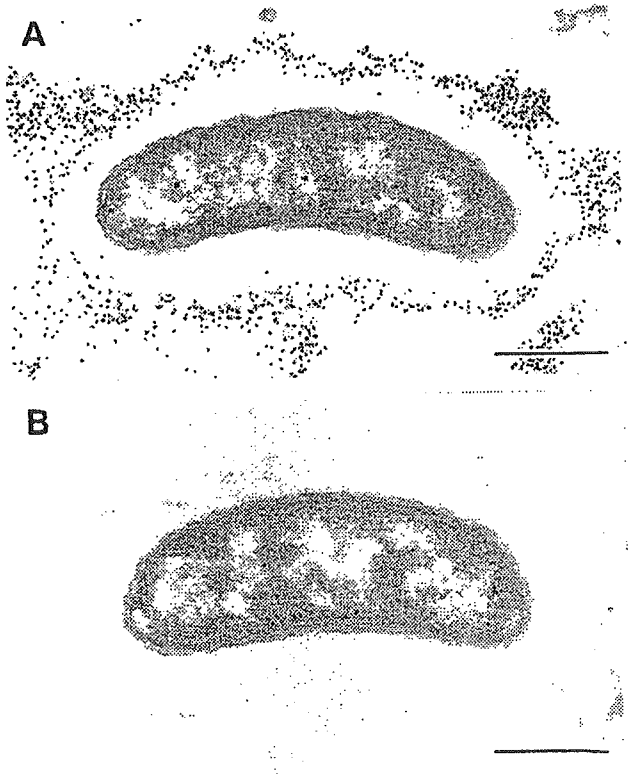

Figure 6. Immunoelectron micrographs of the surface labeling of *V. cholerae* O139 rugose (A) and smooth (B) strains with antiserum against EPS of rugose *V. cholerae* O1. Bars, 0.5 μm.

rugose cells and at the intercellular spaces (Fig. 6A). *V. cholerae* O139 is replacing O1 strains in some areas, and it has been suggested that the O139 strain may cause the eighth cholera pandemic (64, 65). *V. cholerae* O139 Bengal is the second most common etiologic agent of cholera, and the disease caused by this organism has now become endemic in the Indian subcontinent and neighboring countries (66). Prior infection with *V. cholerae* O1, the traditional causative agent of cholera, does not cross-protect against infection with *V. cholerae* O139 (67, 68), since the LPS antigens of the two vibrios are different (44). Vaccines against O1 strains have been developed and are being tested in field trials (69, 70), and they do not cross-protect against *V. cholerae* O139 infection. In our study, interestingly, antiserum against the EPS of *V. cholerae* O1 showed a cross-reaction with EPS materials on the surface of rugose *V. cholerae* O139. We suggest that the study of the genes encoding the EPS (slime) in *V. cholerae* O1 and O139 may facilitate the development of vaccines effective against both *V. cholerae* O1 and O139.

Viable but nonculturable (VBNC) state

A bacterium in the VBNC state is defined as a cell which can be demonstrated to be metabolically active, while beeing incapable of undergoing the sustained cellular division requied for growth in or on a medium normally supporting growth of the cell (28). A variety of environmetal parameters have been reported to induce the entry of various bacteria into the VBNC state (71). Bacteria in VBNC state have been a major concern in public health risk assessment since many pathogenic gram-negative bacteria, such as *V. cholerae, Vibrio vulnificus, V. parahaemolyticus*, and *E. coli* has been shown to enter a VBNC state from which they escape detection and are able to resuscitate to the infectious state following, for example temperature upshift or animal passage (72-74). However, the concept of the VBNC state as a programmed and adaptive response to nutrient starvation has been controversial (29), and another model suggests that cells become nonculturable due to cellular deterioration and, consequently, are moribund (75-77).

The frequency of isolation of *V. cholerae, V. vulnificus,* and *V. parahaemolyticus* is much lower during the winter than the summer months (78). Colwell et al. (79), however, showed that the organism was present and viable in several aquatic environments from which it could not be cultured. This decrease in culturability is thought to arise from the entrance of these bacteria into a VBNC state (28, 80), as has now been shown for at least 30 bacterial species (80). It is thought that the VBNC state may represent a survival response by non-sporulation bacteria exposed to potentially injurious environmental conditions (28). The stresses of low nutrient and low temperature have been shown to be the main causes of the induction of VBNC stages in some pathogenic bacteria (28, 81). In *V. parahaemolyticus, V.*

cholerae and *V. vulnificus*, the VBNC state is induced by a temperature downshift and resuscitation from this state has been reported after the removal of the temperature stress (80-83). Whether true resuscitation of such cells occurs, however, has long been debated. While Whitesides and Oliver (83) have concluded that VBNC cells do indeed leave this dormant state on removal/reversal of the inducing factor, others have suggested that the culturable population which appears is a result of regrowth of one or more culturable cells in the population which had not been detected when the population was assayed (80-82). Resuscitation procedures performed in liquid media, thus, always evoke much controversy. We have tried to resuscitate several VBNC pathogenic bacteria using different methods other than temperature upshift, and will discuss the physiology of VBNC bacteria.

V. cholerae

V. cholerae is a pathogen causing severe diarrheal disease and its infection sources are surface water of natural environments such as rivers, ponds and wells. In epidemic areas, however, detection of *V. cholerae* from natural environment is not always successful (84, 85). Colwell and co-workers have suggested that *V. cholerae* can enter VBNC state (1).

V. cholerae was introduced into VBNC state after 25 days incubation in low nutrient medium at 15°C. 0.1 ml of the VBNC bacterial suspension was heated at 45°C for 1 min and subsequently plated onto a nutrient agar plate. More than 1,000 colonies were recovered after heat-shock treatment (Table 1). After very short period of heat shock treatment, true resuscitation of VBNC cells was observed but not merely regrowth of a few culturable cells remaining in the starting culture (86). Kell et al. (29) reviewed 31 reports about resuscitaion and considered in only three cases, there is sufficient evidence for existence of a reversible state of nonculturablility in nonsporulating bacteria: resuscitation of *Micrococcus luteus* in the presence of a factor produced by viable bacteria and measured using the MPN assay (87), the conversion of

Table 1. Resuscitation of *V. cholerae* from VBNC state by heat shock

Days	Plate count	After heat shock
25	0	5.0×10^3
30	0	2.3×10^3
35	0	0.15×10^3
86	0	6.0×10^3

0.1 ml of the VBNC bacterial culture, with or without heat shock treatment, was plated on L-agar plate and incubated at 37℃ overnight.

nonculturable *V. cholerae* to platable cells via a short heat shock (86), and *Campylobacter jejuni* resuscitated under MPN conditions (88). The mechanism of heat shock on resuscitation remains to be clarified. When bacteria are exposed to high temperatures, a set of proteins is induced to transiently respond to the environmental changes (89). Some of those proteins may participate in the new growth of *V. cholerae*.

V. parahaemolyticus

Since it was first isolated in 1950 during a food-poisoning outbreak in Osaka, Japan, *V. parahaemolyticus* has been shown to be widely distributed in natural aquatic environments around the world (90) and is a well-known food-borne pathogen causing gastrointestinal disease (91).

Starved *V. parahaemolyticus* cells at 4°C reached the nonculturable stage in about 12 days. The true resuscitation of nonculturable cells of *V. parahaemolyticus* occurred after spreading them onto an agar medium supplemented with H_2O_2-degrading compounds such as catalase or sodium pyruvate (92). The response of the *V. parahaemolyticus* following its incubation in starvation medium at 4°C is shown in Fig. 7. Total cell counts remained constant throughout the 16-day period, while colony counts declined

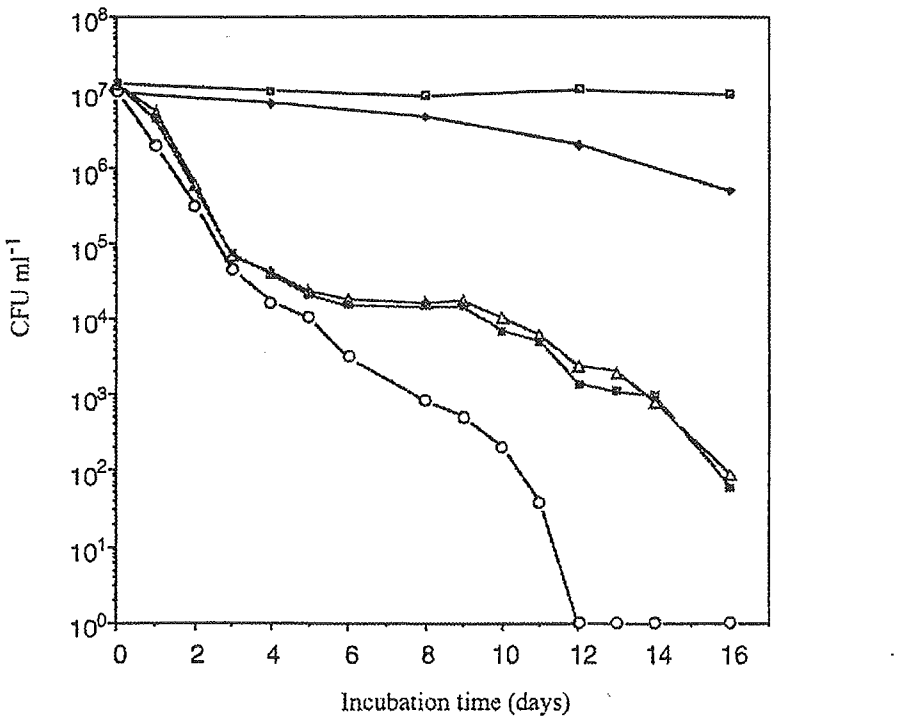

Figure 7. Culturability of *V. parahaemolyticus* incubated at 4°C in the starvation medium (○) and recovery of microcosm samples after spreading on the plates supplemented with catalase (Δ) or pyruvate (■). (⊡), Total cell counts determined by AODC; ♦, viable counts determined by the Live/Dead staining.

to less than 1 CFU ml^{-1} within 12 days. Despite this decline in colony counts, a significant population of viable cells determined by the Live/Dead staining method remained. After development of the nonculturable state, 0.1 ml specimens of the starvation microcosms were spread onto LB agar-3% NaCl plates supplemented with catalase or sodium pyruvate. After incubation at 37°C for 24 h, nonculturable *V. parahaemolyticus* cells produced a number of colonies with 2.3 x 10^3 CFU ml^{-1} on the plates supplemented with catalase, while no colony was observed on the nonamended plates (Fig. 8, at day 12). The number of colony counts of *V. parahaemolyticus* cells on catalase- or pyruvate-amended plates were always higher than those of nonamended plates. Scanning electron microscopy showed that late exponential phase cells of *V. parahaemolyticus* were normally rod shaped with relatively smooth surface (Fig. 9A). In the VBNC state, starved cells formed blebs on the surface of the rod shaped cells (Fig. 9B). Thin sectioning electron micrographs of late exponential phase *V. parahaemolyticus* cells revealed the typical rod shaped cells with a uniform distribution of electron-dense ribosomal and nucleic acid material through out the cytoplasm (Fig. 9C). Thin section micrograph of nonculturable *V. parahaemolyticus* cells (Fig. 9D) showed that a gap was formed between the outer- and the inner-membrane in some parts. The membrane blebs were observed on some part of the outer membrane. Some cells contained less internally staining material and probably lost integrity. These cells were probably dead (Fig. 9D). Starved cells of *V. haemolyticus* showed the formation of blebs on the surface of the cells. Similar membrane vesicles were observed in other marine organisms, such as *Vibrio* sp. strain Ant-300 and *V. cholerae* during the early stage of starvation (93-95). In the study of Baker *et al.* (96), those cells with a detached cell membrane were viable and responded to nutrient addition as quickly as young healthy cells. The blebs could be formed by pieces of cell envelope. During starvation,

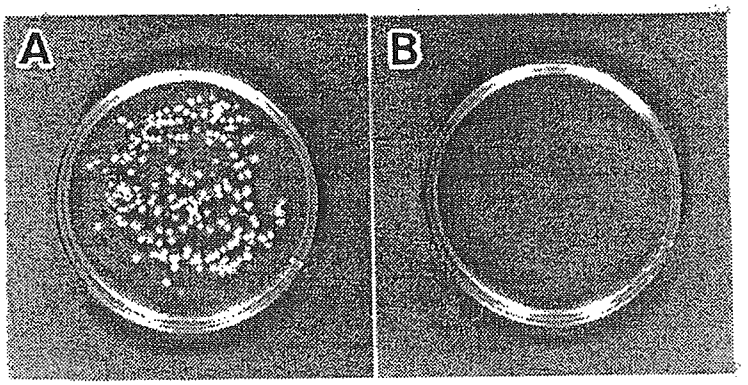

Figure 8. Colonies appeared after resuscitation of nonculturable cells of *V. parahaemolyticus*. Samples (0.1ml) from nonculturable microcosm on day 12 were inoculated on agar amended with catalse (A) or nonamended control (B).

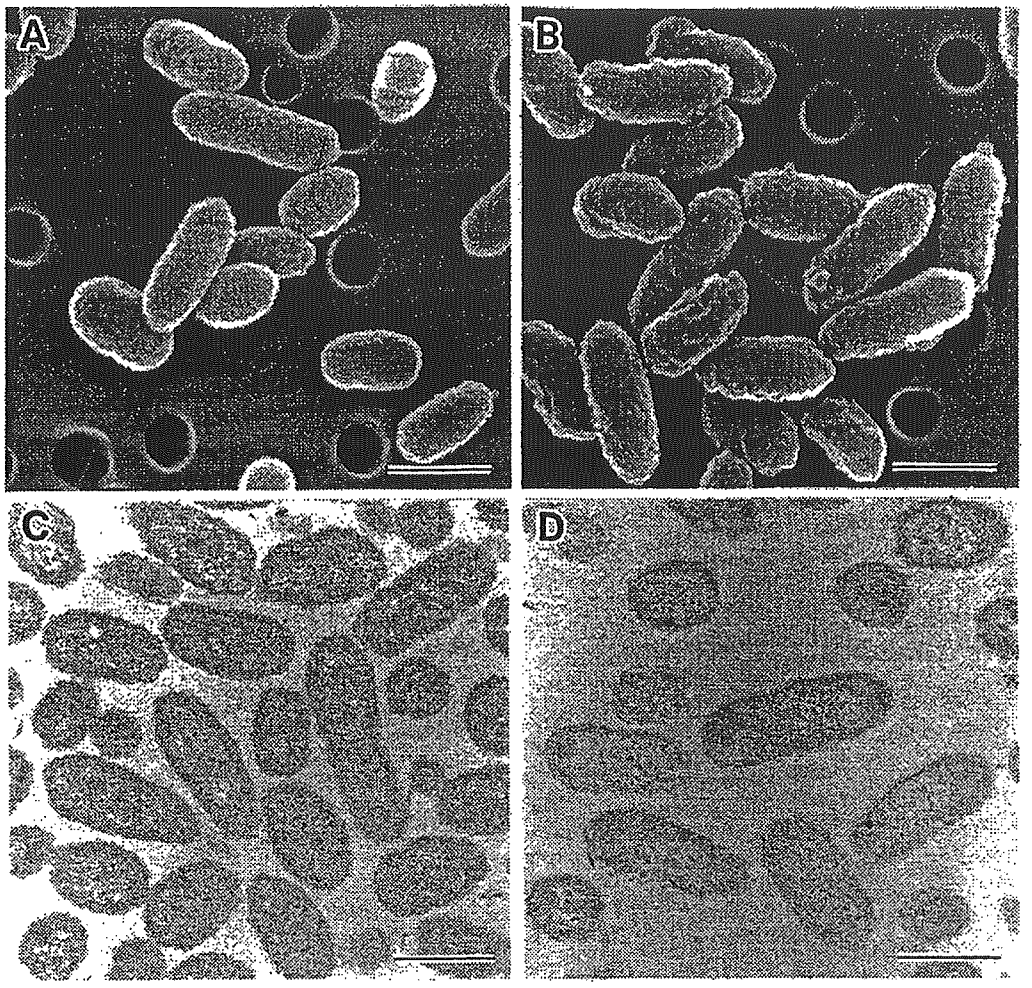

Figure 9. Electron micrographs of *V. parahaemolyticus*. Scanning electron micrograph of late exponential phase cells (A) and cells starved for 12 days at 4°C (B). Thin section electron micrograph of late exponential phase cells (C) and cells starved for 12 days (D). Bars, 1 μm.

excess pieces of cell envelope material may be formed and, in fact, may allow cell volume adjustment via bleb formation. In previous studies, it has been shown that starved cells tend to be small (97, 98). It seems that cell size reduction and bleb formation during starvation is a survival strategy for minimizing cell maintenance requirements and enhancing substrate uptake due to a high surface-to-volume ratio.

Several investigators reported that the viable but nonculturable *Vibrio* species were rounded or spheroid (81, 82, 99). The electronmicrograph of the rounded nonculturable *V. vulnificus* cells (99) showed that the cells maintained a normal cytoplasmic membrane, but possessed a significantly reduced density of ribosomal and nucleic acid material. We suppose that these rounded cells were probably dead. Some studies have argued that resumption of culturability

following a temperature upshift can be accounted for by the regrowth of a few residual culturable cells remaining among the nonculturable population (81, 82, 100). Bogosian et al. (101) demonstrated that the reported resuscitation of VBNC cells during incubation at high temperatures was the result of growth of a small fraction of already culturable, but initially hydrogen peroxide-sensitive, cells. Moreover, there is no evidence for the reductive division exhibited by cells entering the starvation state (6, 79, 102). Taken together, the majority of the nonculturable population prepared in these works (81, 82, 99, 100) does not seem to be alive, then there is no evidence that the rounded cells observed in nonculturable preparations are really viable.

It has been reported that *V. parahaemolyticus* induced into the nonculturable state by low temperature and starvation showed an increased resistance to heating, sonication, low salinity, low pH, or storage at −30°C (82, 103). The VBNC state may make pathogenic *Vibrio* species more resistant to conventional food-processing methods. Besides, virulence may not disappear in VBNC bacteria (79). Seafood is often stored under conditions that induce the VBNC state in *Vibrio* (94). Overreliance on conventional agar plate methods for the microbial inspection of seafoods carries the risk of generating false negative VBNC *Vibrio* species.

Enterohemorrhagic *E. coli* (EHEC)

Enterohemorrhagic *E. coli* (EHEC) is increasingly recognized as a common cause of both epidemic and sporadic disease, notably bloody diarrhea and hemolytic-uremic syndrome. Outbreaks of EHEC infection posed serious health threats in 1996 in Japan (104). Common sources of outbreaks have been traced to the consumption of contaminated hamburger, other foods, and drinking water (105-107). One outbreak-associated isolate of *E. coli* O157:H7 remained viable in tap water for more than a month (108). Furthermore, the unusually prolonged outbreak suggests the survival of these organisms in lake water or repeated contamination but it was not possible to recover the pathogen from the water samples (108). However, this failure to recover the pathogen may result not because of the absence of the organism, but because the cells entered into VBNC state.

Late-exponential-phase cells of *E. coli* O157 became nonculturable in sterilized distilled water microcosms at 4°C. Plate counts declined from 3×10^6 to less than 1 CFU ml^{-1} in about 21 days. However, when samples of microcosms at 21 days were inoculated onto an agar medium amended with catalase or nonenzyme peroxide-degrading compounds such as sodium pyruvate or α-ketoglutaric acid, plate counts increased to 10^4-10^5 CFU ml^{-1} (Fig. 10) (Table 2). Like *V. parahaemolyticus*, morphological changes occurred after the cells entered into nonculturable state. Before starvation, late exponential-phase cells of *E. coli* O157 were normally rod shaped with

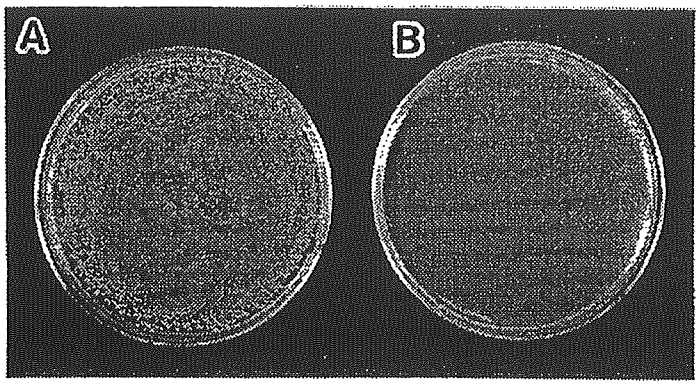

Figure 10. Colonies appeared after resuscitation of nonculturable *E. coli* O157. Sample (0.1ml) from nonculturable microcosm were inoculated on agar amended with catalase (A) or nonamended control (B).

Table 2. Effect of supplements in medium on resuscitation of nonculturable *E. coli* O157 cells. Before resuscitation, plate counts of all samples yielded 0 CFU ml^{-1}.

Supplements for resuscitation	Plate counts (CFU ml^{-1}) After resuscitation
Catalase (2,000U/plate)	1.1×10^5
Sodium pyruvate (0.1%)	2.1×10^4
α-ketoglutalic acid (0.1%)	1.8×10^4
3, 3'-Thiodipropionic acid (0.1%)	4.8×10^4
Heat-denatured catalase*	0

* Originally corresponding to 2000 U/plate of catalase.

relatively smooth surface (Fig. 11A). In nonculturable state, rod-shaped cells appeared to be shorter than that of late exponential-phase cells. The formation of blebs was also observed on the surface of the nonculturable cells (Fig. 11B). Thin-section micrographs of *E. coli* O157 cells (Fig. 11C and D) showed that some parts of the outer membrane of nonculturable cells were separated and a gap was formed between it and the inner membrane.

The infectious dose of *E. coli* O157 is believed to be very low (109). Most of the bacterial populations are probably in the nonculturable state and it seems likely that this state hampers the recovery of the pathogen from the contaminated food and water. This probably explains why the infectious dose of *E. coli* O157 infection is very low. Monitoring disease patterns using laboratory reports is a useful way of looking for major trends, but might be prone to major biases due to under-detection of nonculturable enteric pathogens by routine laboratory methods.

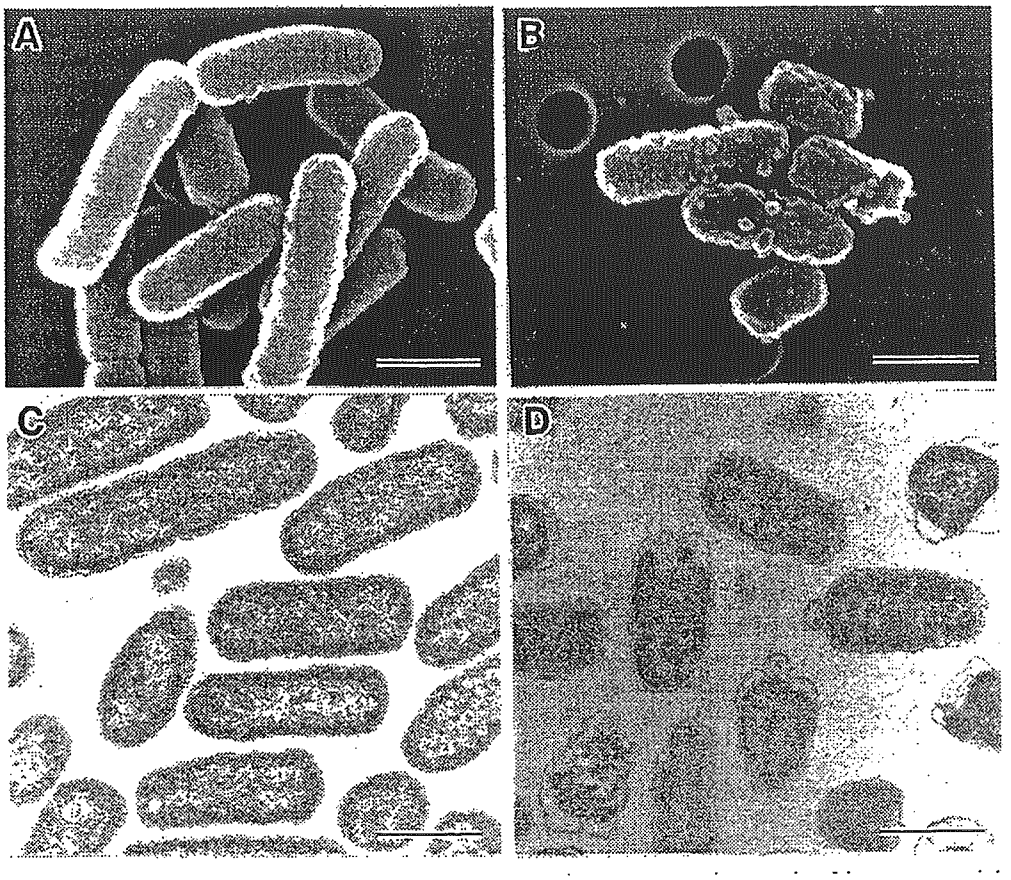

Figure 11. Electron micrographs of *E. coli* O157. Scanning electron micrograph of late exponential phase cells (A) and cells starved for 3 weeks (B). Thin sectioning electron micrograph of late exponential phase cells (C) and cells starved for 3 weeks (D). Bars, 1 μM.

We have demonstrated the resuscitation of VBNC cells of several pathogens including *V. parahaemolyticus* (92), EHEC (110) and *Aeromonas hydrophila* (111) by inoculating onto an agar medium amended with catalase or nonenzyme peroxide-degrading compounds such as sodium pyruvate or α-ketoglutaric acid. We eliminated the incubation process after a temperature upshift of VBNC cell suspension in which regrowth might have occurred, so that the colonies emerging on the supplemented plates are regarded as a consequence of true resuscitation. It is proposed that a sudden transfer of injured or starved cells to nutrient-rich agar at temperatures optimal for enzyme activities initiates an imbalance in metabolism, inducing a near instantaneous production of superoxide and free radicals. In the absence of phenotypic adaptation, the cells are not equipped to detoxify reactive oxygen intermediates and, as a result, a proportion or all of these cells die (112). Our results indicate that an efficient protection against oxidative stress during bacterial inoculation is of fundamental importance in improved recovery of nonculturable cells.

Conclusion

The adaptations that bacteria undergo under stressful conditions are not limited to a few aspects of the cell but rather involve global changes in cell physiology. It is evident that biofilm formation is a key factor for survival in diverse environments. We have shown that *V. cholerae* undergo phase variation to a rugose colony morphology associated with the expression of an amorphous exopolysaccharide that promotes biofilm formation, and we also indicated that rugose strains displayed resistance to osmotic and oxidative stress. Recent advances have led to our current definition of a bacterial biofilm as a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface. Biofilms constitute a protected mode of growth that allows cells to survive in hostile environments and also disperse to colonize new niches (113).

VBNC state has also been proposed to represent a survival strategy in response to adverse environmental conditions (113). However, it has long been debated whether pathogens exist in VBNC state. The VBNC hypothesis argues for nonculturable cells beeing genetically programmed survival forms awaiting appropriate conditions for re-growth. If VBNC formation is a physiological adaptation similar to spore formation, the mutants that fail to enter VBNC state could be selected (114). No such mutants have been reported. Moreover, almost all published studies fail to discriminate adequately between resuscitation and regrowth of any culturable cells initially present in the population. To eliminate the possibility of such regrowth during resuscitation, we have tried to find new resuscitation methods other than temperature upshift or incubation in some liquid media. In our studies (92, 110, 111), a direct inoculation on the plates amended with hydrogen peroxide-degrading compounds promoted the recovery of several nonculturable pathogens. Our finding was followed by the report of nonculturable *V. vulunificus* (101). We assume that starved nonculturable cells can not be recovered when suddenly transferred to rich medium because of their susceptibility to oxidative stress. We consider such hydrogen peroxide-sensitive condition of cells to be a part of the physiological states of VBNC cells.

Further analysis of molecular mechanisms related to bacterial life cycle including biofilm formation and VBNC state is still of the utmost importance to public health.

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References

1. Colwell, R.R., and Huq. A.1994, *Vibrio cholerae* and Cholera, T. Kaye (Ed.), ASM Press, Washington D.C., 117.
2. Jefferson, K.K. 2004, FEMS Microbiol. Lett., 236, 163.
3. Poindexter, J. S. 1981, Microbiol. Rev., 45, 123.
4. Wrangstadh, M., Szewzyk, U., Ostling, J., and Kjelleberg, S. 1990, Appl. Environ. Microbiol., 56, 2065.
5. Dawson, M. P., Humphrey, B., and Marshall, K.C. 1981, Curr. Microbiol., 6, 195.
6. Kjelleberg, S., Hermansson, M., Maden, P., and Jones, G.W. 1987, Annu. Rev. Microbiol., 41, 25.
7. Kjelleberg, S., Humphrey, B.A., and Marshall, K.C. 1982, Appl. Environ. Microbiol., 43, 1166.
8. Maden, P., Nystrom, T., and Kjelleberg, S. 1987, FEMS Microbiol. Ecol., 45, 233.
9. Maden, P., Tunlid, A., Malmcrona-Friberg, K., Odham, G., and Kjelleberg, S. 1985, Arch. Microbiol., 149, 326.
10. Matin, A., Auger, E.A., Blum, P.H., and Schultz, J.E. 1989, Annu. Rev. Microbiol., 42, 293.
11. Henderson, I.R., Owen, P., and Nataro, J.P. 1999, Mol. Microbiol., 33, 919.
12. Wai, S.N., Mizunoe, Y., Takade, A., Kawabata, S., and Yoshida, S. 1998, Appl. Environ. Microbiol., 64, 3648.
13. Mizunoe, Y., Wai, S.N., Takade, A., and Yoshida, S. 1999, Infect. Immun., 67, 958.
14. Yildiz, F.H., and Schoolnik, G.K. 1999, Proc. Natl. Acad. Sci. USA., 96, 4028.
15. Enos-Berlage, J.L., and McCarter, L.L. 2000, J. Bacteriol., 182, 5513.
16. McCarter, L.L. 1998, J. Bacteriol., 180, 3166.
17. McCarter, L.L. 1999, J. Mol. Microbiol. Biotechnol., 1, 51.
18. Alliston, D.G., and Sutherland. 1984, J. Microbiol. Methods., 2, 93.
19. Costerton, J.W., and Lashen, E.S. 1984, Mater. Prot. Perf., 23, 34.
20. Costerton, J.W., Cheng, K.J., Geesey, G.G., Ladd, T.I., Nickel, J.C., Dasgupta, M., and Marrie, T.J. 1987, Annu Rev Microbiol., 41, 435.
21. Dickinson, G.M., and Bisno, A.L. 1989, Antimicrob. Agents Chemother., 33, 597.
22. Dickinson, G.M., and Bisno, A.L. 1989, Antimicrob. Agents Chemother., 33, 602.
23. Marrie, T.J., and Costerton. 1985, J. Clin. Microbiol., 22, 924.
24. Anderson, G.G., Palermo, J.J., Schilling, J.D., Roth, R., Heuser, J., and Hultgren, S.J. 2003, Science, 301, 105.
25. Brown, M.R.W., and Williams, P. 1985, Annu. Rev. Microbiol., 39, 527.
26. Justice, S.S., Hung, C., Theriot, J.A., Fletcher, D.A., Anderson, G.G., Footer, M.J., and Hultgren, S.J. 2004, Proc. Natl. Acad. Sci. USA., 101, 1333.

27. Barer, M.R., Gribbon, L.T., Harwood, C.R., and Nwoguh, C.E. 1993, Rev. Med. Microbiol., 4, 183.
28. Oliver, J.D. 1993, Starvation in Bacteria, S. Kjelleberg (Ed.), Plenum Press, New York, 239.
29. Kell, D.B.A., Kaprelyants, S., Weichart, D.H., Harwood, C.R., and Barer, M.R. 1998, Antonie Leeuwenhock, 73, 169.
30. Barer, M.R. and Harwood, C.R. 1999, Adv. Microb. Physiol., 41, 93.
31. Rahman, I., Shahamat, M., Kirchman, P.A., Rissek-Cohen, E., and Colwell, R.R. 1994, Appl. Environ. Microbiol., 60, 3573.
32. Lleò, M.M., Tafi, M.C., and Canepari, P. 1998, Syst. Appl. Microbiol. 21, 333.
33. Lloyd, D. and Hayes, A.J. 1995, FEMS Microbiol. Lett., 133, 17.
34. Lleò, M.M., Pierobon, S., Tafi, M.C., Signoretto, C., and Canepari, P. 2000, Appl. Environ. Microbiol., 66, 4564.
35. Davey, M.E., and O'Toole G.A. 2000, Microbiol. Mol. Biol. Rev., 64, 847.
36. O'Toole, G., Kaplan, H.B., and Kolter, R. 2000, Annu. Rev. Microbiol., 54, 49.
37. Davey, M.E., Caiazza, N.C., and O'Toole, G.A. 2003, J. Bacteriol., 185, 1027.
38. Davies, D.G., Parsek, M.R., Pearson, J.P., Iglewski, B.H., Costerton, J.W., and Greenberg, E.P. Science, 1998, 280, 295.
39. Berche, P., Poyart, C., Abachin, E., Lelievre, H., Vandepitte, J., Dodin, A., and Fournier, J.M. 1994, J. Infect. Dis., 170, 701.
40. Faruque, S.M., Alim, A.A.R., Roy, S.K., Khan, F., Nair, G.B., Sack, R.B., and Albert, M.J. 1994, J. Clin. Microbiol., 32, 1050.
41. Johnson, J.A., Salles, C.A., Panigrahi, P., Albert, M.J., Wright, A.C., Johnson, R.J., and Morris, J.G. Jr. 1994, Infect. Immun., 62, 2108.
42. Waldor, M.K., and Mekalanos, J.J. 1994, J. Infect. Dis., 170, 278.
43. Comstock, L.E., Johnson, J.A., Michalski, J.M., Morris, J.G. Jr., and Kaper, J.B. 1996, Mol. Microbiol., 19, 815.
44. Hisatsune, .K, Kondo, S., Isshiki, Y., Iguchi, T., Kawamata, Y., and Shimada, T. 1993, Biochem. Biophys. Res. Commun., 196, 1309.
45. Jonson, G., Osek, J., Svennerholm, A.M., and Holmgren, J. 1996, Infect. Immun., 64, 3778.
46. Comstock, L.E., Maneval, D. Jr., Panigrahi, P., Joseph, A., Levine, M.M., Kaper, J.B., Morris, J.G. Jr., and Johnson, J.A. 1995, Infect. Immun., 63, 317.
47. Waldor MK, Colwell R, Mekalanos JJ. 1994, Proc. Natl. Acad. Sci. USA., 1994, 91, 11388.
48. White, P.B. 1938, J. Pathol. Bacteriol., 46, 1.
49. Rice, E.W., Johnson, C.J., Clark, R.M., Fox, K.R., Reasoner, D.J., Dunnigan, M.E., Panigrahi, P., Johnson, J.A., and Morris, J.G. Jr. 1992, Lancet. 340, 740.
50. Morris, J.G. Jr., Sztein, M.B., Rice, E.W., Nataro, J.P., Losonsky, G.A., Panigrahi, P., Tacket, C.O., and Johnson, J.A. 1996, J. Infect. Dis., 174, 1364.
51. Ali, A., Rashid, M.H., and Karaolis, D.K. 2002, Appl. Environ. Microbiol., 2002, 68, 5773.
52. Watnick, P.I., Lauriano, C.M., Klose, K.E., Croal, L., and Kolter, R. 2001, Mol. Microbiol., 39, 223.
53. Danese, P.N., Pratt, L.A., and Kolter, R. 2000, J. Bacteriol., 182, 3593.
54. Davies, D.G., Chakrabarty, A.M., and Geesey, G.G. 1993, Appl. Environ. Microbiol., 59, 1181.

55. Hoyle, B.D., Williams, L.J., and Costerton, J.W. 1993, Infect. Immun., 61, 777.
56. Mulvey, M.A., Schilling, J.D., and Hultgren, S.J. 2001, Infect. Immun., 69, 4572.
57. Yildiz, F.H., Dolganov, N.A., Schoolnik. G.K. 2001, J. Bacteriol., 183, 1716.
58. Casper-Lindley, C., and Yildiz, F.H. 2004, J. Bacteriol., 186, 1574.
59. Hammer, B.K., and Bassler, B.L. 2003, Mol. Microbiol., 50, 101.
60. Jobling, M.G, and Holmes, R.K. 1997, Mol. Microbiol., 26, 1023.
61. Zhu, J., and Mekaranos, J.J. 2003, Dev. Cell, 5, 647.
62. Haugo, A.J., and Watnick, P.I. 2002, Mol. Microbiol., 45, 471.
63. Yildiz, F.H., Liu, X.S., Heydorn, A., and Schoolnik, G.K. 2004, Mol. Microbiol., 53, 497.
64. Garg, S., Saha, P.K., Ramamurthy, T., Deb, B.C., Nair, G.B., Shimada, T., and Takeda, Y. 1993, J. Infect., 27, 108.
65. Jesudason, M.V., and John, T.J. 1993, Lancet, 341, 1090.
66. Albert, M.J. 1996, Indian J. Med. Res., 104, 14.
67. Albert, M.J., Alam, K., Ansaruzzaman, M., Qadri, F., and Sack, R.B. 1994, J. Infect. Dis., 169, 230.
68. Albert, M.J., Siddique, A.K., Islam, M.S., Faruque, A.S., Ansaruzzaman, M., Faruque, S.M., and Sack, R.B. 1993, Lancet, 341, 704.
69. Levine, M.M., and Tacket, C.O. 1994, *Vibrio cholerae* and cholera, I.K. Wachsmuth (Ed.), ASM Press, Washington D.C., 395.
70. Holmgren, J., Osek, J., and Svennerholm, A.-M. 1994, *Vibrio cholerae* and cholera, I.K. Wachsmuth (Ed.), ASM Press, Washington D.C., 415
71. Gauthier, M.J. 2000, Nonculturable Microorganisms in the Environment, R.R. Colwell, and D.J. Grimes (Ed.), ASM Press, Washington D.C., 87.
72. Oliver, J.D. 2000, Nonculturable Microorganisms in the Environment, R.R. Colwell, and D.J. Grimes (Ed.), ASM Press, Washington D.C., 277.
73. Huq, A., Rivera, I.N.G., and Colwell, R.R. 2000, Nonculturable Microorganisms in the Environment, R.R. Colwell, and D.J. Grimes (Ed.), ASM Press, Washington D.C., 301.
74. Colwell, R.R. 2000, Nonculturable Microorganisms in the Environment, R.R. Colwell, and D.J. Grimes (Ed.), ASM Press, Washington D.C., 325.
75. Dukan, S., and Nystrom, T. 1999, J. Biol. Chem., 274, 26027.
76. Bogosian, G., Bourneuf, E.V. 2001, EMBO. Rep., 2, 770.
77. Desnues, B., Cuny, C., Gregori, G., Dukan, S., Aguilaniu, H., and Nystrom, T. 2003, EMBO. Rep., 4, 400.
78. Chowdhury, M.A., Yamanaka, H., Miyoshi, S., and Shinoda, S. 1990, FEMS. Microbiol. Ecol., 74, 1.
79. Colwell, R.R., Brayton, P.R., Grimes, D.J., Roszak, D.B., Huq, S.A., and Palmer, L.M. 1985, Bio/Technology, 3, 817.
80. Oliver, J.D. 1995, FEMS. Microbiol. Lett., 133, 203.
81. Ravel, J., Knight, I.T., Monahan, C.E., Hill, R.T., and Colwell, R.R. 1995, Microbiology, 141, 377.
82. Jiang, X., and Chai, T.J. 1996, Appl. Environ. Microbiol., 62, 1300.
83. Whitesides, M.D., and Oliver, J.D. 1997, Appl. Environ. Microbiol., 63, 1002.
84. Brayton, P.R., Tamplin, M.L., Huq, A., and Colwell, R.R. 1987, Appl. Environ. Microbiol., 53, 2862.
85. Huq, A., Colwell, R.R., Rahman, R., Ali, A., Chowdhury, M.A., Parveen, S., Sack, D.A., Russek-Cohen, E. 1990, Appl. Environ. Microbiol., 56, 2370.

86. Wai, S.N., Moriya, T., Kondo, K., Misumi, H., and Amako, K. 1996, FEMS. Microbiol. Lett., 136, 187.
87. Kaprelyants, A.S., Mukamolova, G.V., and Kell, D.B. 1994, FEMS.Microbiol. Lett., 115, 347.
88. Bovill, R.A., and Mackey, B.M. 1997, Microbiology, 143, 1575.
89. Yura, T., Nagai, H., and Mori, H. 1993, Annu. Rev. Microbiol., 47, 321.
90. Davis, J.W., and Sizemore, R.K. 1982, Appl. Environ. Microbiol., 43, 1092.
91. Chai, T., and Pace, J. 1993, Foodborne Disease Handbook, Y.H. Hui, J.R. Gorham, and K.D. Murell. (Ed.), Marcel Dekker, New York, 395.
92. Mizunoe, Y., Wai, S.N., Ishikawa, T., Takade, A., and Yoshida, S. 2000, FEMS. Microbiol. Lett., 186, 115.
93. Hood, M.A., Guckert, J.B., White, D.C., and Deck, F. 1986, Appl. Environ. Microbiol., 52, 788.
94. Marden, P., Tunlid, A., Malmcrona-Friberg, A., Odham, G. and Kjelleberg, S. 1985, Arch. Microbiol., 142, 326.
95. Oliver, J.D., Nilsson, L. and Kjelleberg, S. 1991, Appl. Environ. Microbiol., 57, 2640.
96. Baker, R.M., Singleton, F.L. and Hood, M.A. 1983, Appl. Environ. Microbiol., 46, 930.
97. Morita, R.Y. 1993, Starvation in Bacteria, S. Kjelleberg (Ed.), Plenum Press, New York, 1.
98. Roszak, D.B., and Colwell, R.R. 1987, Microbiol. Rev., 51, 365
99. Linder, K. and Oliver, J.D. 1989, Appl. Environ. Microbiol., 55, 2837.
100. Weichart, D., Oliver, J.D. and Kjelleberg, S. 1992, FEMS Microbiol. Lett., 79, 205.
101. Bogosian, G., Aardema, N.D., Bourneuf, E.V., Morris, P.J., and O'Neil, J.P. 2000, J. Bacteriol., 182, 5070.
102. Novitsky, J.A., and Morita, R.Y. 1976, Appl. Environ. Microbiol., 32, 617.
103. Wong, H.C., and Wang, P. 2004, J. Appl. Microbiol., 96, 359.
104. National Institute of Health Japan. 1996, Infect. Agents. Surveillance. Rep., 17, 180.
105. Ostroff, S.M., Griffin, P.M., Tauxe, R.V., Shipman, L.D., Greene, K.D., Wells, J.G., Lewis, J.H., Blak, P.A., and Kobayashi, J.M. 1990, Am. J. Epidemiol., 132, 239.
106. Belongia, E.A., MacDonald, K.L., Parham, G.L., White, K.E., Korlath, J.A., Lobato, M.N., Strand, S.M., Casale, K.A., and Osterholm, M.T. 1991, J. Infect. Dis., 164, 338.
107. Swerdlow, D.L., Woodruff, B.A., Brady, R.C., Griffin, P.M., Tippen, S., Donnell, H.D. Jr., Geldreich, E., Payne, B.J., Meyer, A. Jr., and Wells. J.G. 1992, Ann. Intern. Med., 117, 812.
108. William, E.K., McAnulty, J.M., Hoesly, F.C., William, L.P., Hedberg, K., Oxman, G.L., Barrett, T.J., Pfaller, M.A., and Fleming, D.W. 1994, New Engl. J. Med., 331, 579
109. Griffin, P.M., and Tauxe, R.V. 1991, Epidemiol. Rev., 13, 60.
110. Mizunoe, Y., Wai, S.N., Takade, A., and Yoshida, S. 1999, Arch. Microbiol., 172, 63.
111. Wai, S.N., Mizunoe, Y., Takade, A., and Yoshida, S. 2000, Arch. Microbiol., 173, 307.

112. Bloomfield, S.F., Stewart, G.S., Dodd, C.E., Booth, I.R. and Power, E.G. 1998, Microbiology 144, 1.
113. Hall-Stoodley, L., Costerton, J.W., and Stoodley, P. 2004, Nat, Rev. Microbiol., 2, 95.
114. Nystrom, T. 2003, BoiEssays, 25, 204.

Novel bactericidal surface: Catechin-loaded surface-erodible polymer prevents biofilm formation

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Abstract: We developed a novel bactericidal surface based on a catechin-loaded surface-erodible polymer. (−)-Epigallocatechin-3-gallate (EGCg), which is the main constituent of tea catechins, showed a dose-dependent inhibitory effect on *Escherichia coli* biofilm formation and a dose-dependent enhanced destructive effect on biofilm. EGCg-immobilized surfaces were prepared by photopolymerization of liquid biodegradable polyesters. The releasing rate was enhanced with an increase in surface-erosion rate of photocured polymers. Polymers with high releasing capacity dose-dependently reduced biofilm formation on the surfaces. The confocal laser scanning microscopic and scanning electron microscopic observations revealed that EGCg induced biofilm-destructing activities, which include bacterial membrane damage, degradation of exopolysaccharides, and detachment of colonized cells. From these results, potential advantages of the clinical use of catechin-loaded polymer-coated implants or catheters are discussed in terms of a reduced occurrence of biomaterial-centered infections without substantial toxicity or adverse effects. © 2005 Wiley Periodicals, Inc. J Biomed Mater Res 75A: 146–155, 2005

Key words: biofilm; catechin; *Escherichia coli*; local delivery; biodegradable polymer

INTRODUCTION

The biomass of bacteria and extracellular materials that accumulate on foreign body substrates is called a biofilm.[1,2] Once biofilm is formed on an artificial implant in a body, it causes symptoms of chills and fever, and in the worst case, becomes life-threatening by leading to septic shock, which is a lethal systemic response to infection. Biofilm is highly resistant to drugs because of its well-stabilized bioarchitecture, which prevents the permeation of drugs into its deeper regions.[3] Therefore, the exchange of infected implants with new ones is often the only solution to various biofilm-related problems.

Various techniques of local administration of cytocidal drugs with the use of drug-eluting biomaterials to prevent biofilm formation on biomaterial surfaces have been reported.[4,5] Two leading sustained releasing systems have been extensively studied: the releases of antibiotic-loaded and silver-impregnated biomaterials. These have been effective in *in vitro* studies, but these drugs often show unfavorable effects when they are used in a clinical setting.[6,7] In some studies, concern has been expressed about the contribution of the use of antibiotic-loaded biomaterials to the development of antibiotic-resistant bacteria.[8] Long-term administration of silver ions to the human body is associated with gray discoloration of the skin.[9]

Recently, catechins, which are polyphenols extracted from daily beverages such as green tea, have been reported to show bactericidal effects against var-

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TABLE I
EGCg MICs Against Gram-Negative and Gram-Positive Bacteria

Strain	MIC (μg/mL)	Reference[a]
Gram-negative		
Escherichia coli (YMel)	250–300	1
Klebsiella pneumoniae	>800	2
Salmonella typhi	>800	2
Gram-positive		
Staphylococcus aureus	50–100	2
Methicillin-resistant *S. aureus*	50–100	2
S. epidermidis	50	2

[a]1, This study; 2, Yoda et al.[10]

ious strains of Gram-positive and Gram-negative bacteria, as a result of damage to the bacterial membrane (Table I).[10,11] Catechins also inhibit the growth of cancer cells and scavenge free radicals.[12,13] Among catechin family substances, (−)-epigallocatechin-3-gallate (EGCg, Fig. 1) in particular, which is the main constituent of tea catechins, exhibits the strongest bactericidal and biological activity.[10,14] The sustained release of EGCg from drug-loaded polymer may be useful for the prevention of biofilm formation without substantial disadvantage to the body if it is coated onto or impregnated into the biomaterial surface.

In this study, we fabricated EGCg-loaded biodegradable polymers, and examined the releasing profiles of EGCg and the dose-dependent effect on biofilm prevention using *Escherichia coli* (*E. coli*), the biofilm of which is a major cause of clogging of biliary stents and urinary catheters.[15,16] Confocal laser scanning microscopy using genetically fluoroprobed *E. coli* and fluorescent staining of dead *E. coli*, and scanning electron microscopy revealed the morphological change of biofilm exposed to EGCg. The potential efficacy and usefulness of catechin-immobilized bactericidal surfaces are discussed.

MATERIALS AND METHODS

Bacterial strain and growth conditions

E. coli YMel, which was transformed to produce green fluorescent protein (GFP) as previously reported,[17] was grown for 18 h at 37°C in modified Luria-Bertani (LB) medium containing 50 μg/mL ampicillin and 3 g/L NaCl, and then scaled up to a concentration of approximately 2×10^8 colony-forming units per milliliter (CFU/mL), which was then diluted for additional experiments.

Susceptibility testing of *E. coli* biofilm to catechin

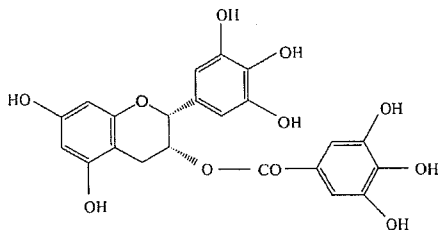
E. coli (2×10^3 CFU/mL) was incubated with a round polyurethane (PU) sheet (obtained from Olympus Optical Co., Ltd., Tokyo, Japan) in modified LB medium with the indicated concentrations (0–0.25 m*M*) of EGCg (Sigma-Aldrich, Inc., St. Louis, MO) at pH 7.4 dissolved in phosphate-buffered saline (PBS) (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), for 24 h at 37°C under static conditions. Subsequently, the viable adhered *E. coli* was counted. However, the effect of EGCg on established biofilm, which was obtained by incubating *E. coli* (2×10^3 CFU/mL) for 24 h in the medium on the PU film, was evaluated after additional incubation in the presence of EGCg at indicated concentrations (0–50 m*M*) for 24 h at 37°C. Then, viable bacterial cell count, confocal laser scanning microscopy, and scanning electron microscopy of the biofilm-formed PU surface were performed. The minimal growth-inhibiting concentration (MIC) of EGCg was also determined by the twofold agar dilution method.[18] The chemical structure of EGCg is shown in Figure 1.

Chemical reagents and photocured materials

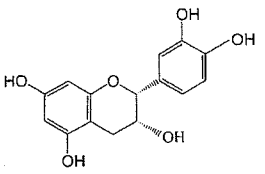
Three photocurable biodegradable liquid prepolymers, TMC/LL/PEG1k, TMC/PEG200, and TMC/TMP, were synthesized according to our previous method.[19] The chemical structures of these copolymers are shown in Figure 2. Briefly, an equimolar mixture of trimethylene carbonate (TMC) and L-lactide (LL) were copolymerized using poly(ethylene glycol) (PEG) with molecular weights of 1000 g/mol or of 200 g/mol or trimethylolpropane (TMP) as an initiator. These liquid prepolymers were mixed with EGCg dissolved in acetone (Sigma-Aldrich). The characteristics (receding water contact angle, water uptake, and surface erosion rate) of photocured polymers, which were cited from our previous report,[19] are shown in Table II.

Catechin release test

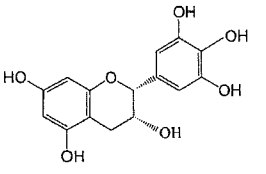
Two hundred fifty milligrams of a liquid mixture of a prepolymer and 5, 10, or 20 weight percent (wt %) of EGCg was coated on the flat bottom (surface area: 1.1 cm^2) of a



(−)-Epigallocatechin-3-gallate (EGCg)



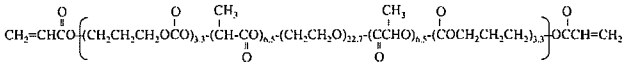
(−)-Epicatechin (EC)



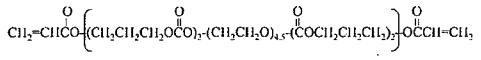
(−)-Epigallocatechin (EGC)

Figure 1. Chemical structures of catechins.

(A) TMC/LL/PEG1k



(B) TMC/PEG200



(C) TMC/TMP

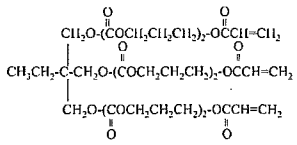


Figure 2. Chemical structures of photocurable biodegradable polymers. TMC, trimethylene carbonate; LL, L-lactide; PEG, poly(ethylene glycol); and TMP, trimethylolpropane.

glass bottle and subsequently irradiated with ultraviolet (UV) light for 5 min, using Spot Cure (SP-V; Ushio, Inc., Yokohama, Japan), to form a solid polymer. Subsequently, 1 mL of PBS was added to each bottle, and the solution was withdrawn at scheduled time intervals. The amount of EGCg was measured according to the Folin-Ciocalteu reagent method[20]: 100 μL of sample solution was introduced in a test tube, 900 μL of PBS, 500 μL of Folin-Ciocalteu reagent (Sigma-Aldrich), and 5 mL of 0.4*M* sodium carbonate (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were added, and the contents were mixed and allowed to stand for 30 min. Absorption at 660 nm was measured in a UV spectrophotometer (DU530; Beckman Instruments, Inc., Fullerton, CA). The amount was expressed as EGCg in milligrams per square centimeter (mg/cm^2) released from the photocured polymer surface, which was calculated using a standard curve generated with indicated concentrations (0–0.5 m*M*) of EGCg. All test points were measured three times.

Bacterial adhesion on catechin-loaded polymers under static conditions

The catechin-mixed liquid prepolymers were coated on PU sheets using a spin coater (Mikasa Co., Ltd., Tokyo, Japan) and then photocured using UV light. Photocured polymer-coated discs (8 mm in diameter) were cut with a belt punch. They were sterilized by ethylene oxide before experiments and stored under dark and anaerobic conditions at room temperature for at least 4 weeks before use. The polymer discs were placed in a 48-well cell culture cluster using sterilized forceps and incubated with *E. coli* (2×10^3 CFU/mL) for 24 h at 37°C under static conditions. Viable bacterial cell count and confocal laser scanning microscopy were performed.

Bacterial adhesion on catechin-loaded polymers under flow conditions

A modified Robbins device (MRD) (Tyler Co., Edmonton, Canada) with 25 sample holders was used to study bacterial adhesion under flow conditions (Fig. 3). The catechin-loaded polymer [poly(TMC/LL/PEG1k)] discs (8 mm in diameter) were glued onto the sample holders with silicone paste and sterilized by ethylene oxide. At first, *E. coli* (2×10^5 CFU/mL)-containing solution was passed through the MRD for 1 h at a flow rate of 1 mL/min. Then, the sterile modified LB medium was passed through the device for 23 h at the same flow rate. The cross-section of the flow through the MRD was rectangular (11 × 3 mm). Polymer discs loaded with lower concentrations of EGCg were placed in upstream of the flow, and polymer discs of higher concentration were placed downstream. We confirmed that there was no significant difference in bacterial adhesion attributable to sample position in a preliminary experiment using plain PU discs. The temperature of the MRD was maintained at approximately 37°C during the experiment. Viable bacterial cell count on the polymer surface was performed.

Viable bacterial cell count on the polymer surface

Bacteria-incubated polymer discs were gently immersed in 2 mL of PBS, and then sonicated for 60 s to detach all the adhered *E. coli*. Serial dilutions of the solution containing the detached *E. coli* were poured onto LB agar plates. After overnight incubation, viable bacterial cells were counted and expressed relative to the surface area of the polymer discs (CFU/cm^2). Experiments were run with three samples.

Confocal laser scanning microscopy

The biofilm grown under static conditions was examined using confocal laser scanning microscopy (Radiance 2000; BioRad, Hercules, CA). After incubating *E. coli* on a polymer disc, culture medium was removed and 300 μL of 60 μ*M* propidium iodide (Molecular Probes, Inc., Eugene, OR), which penetrates only bacteria with damaged membranes and stains nucleic acid with red fluorescence, was gently applied and incubated at room temperature in the dark for 15 min to stain dead *E. coli* cells. Images were recorded at an excitation wavelength of 488 nm and an emission wavelength of 515 ± 30 nm for green fluorescent protein, and an excitation wavelength of 514 nm and an emission wavelength of 600 ± 50 nm for propidium iodide.

TABLE II
Characteristics of Photocured Polymers[a]

Material	Receding Water Contact Angle (°)	Water uptake (%)	Surface Erosion Rate (μm/cm^2/day)
TMC/LL/PEG1k	9.70 ± 1.74	32	4.3
TMC/PEG200	27.0 ± 3.56	1.0	1.4
TMC/TMP	47.3 ± 5.33	0.40	1.1

[a]Cited from our previous article.[19]

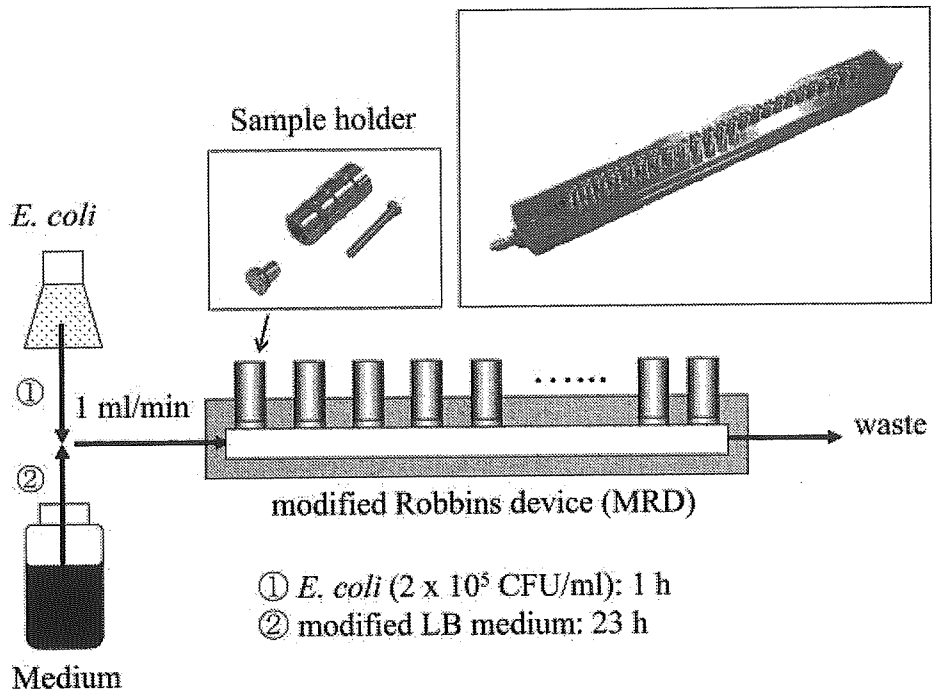


Figure 3. Experimental setup for bacterial adhesion study under flow conditions. *E. coli* suspension and sterile medium are perfused through the modified Robbins device (MRD). The EGCg-loaded polymer (TMC/LL/PEG1k) discs were glued onto the sample holders.

Scanning electron microscopy

The biofilm grown on a glass slide under static conditions was fixed in 2% glutaraldehyde (Electron Microscopy Science, Hatfield, PA) in 0.1*M* phosphate buffer for 1 h at room temperature. The fixed samples were dehydrated for 20 min at each step in an ascending ethanol series, sputter-coated with platinum, and evaluated by scanning electron microscopy (JSM-840A; JEOL, Tokyo, Japan).

Statistical analysis

Statistical analysis was performed with the StatView 5.0 program (Abacus, Berkley, CA). Data are shown as means ± SD. Statistical analysis was performed by using analysis of variance. Differences at $p < 0.05$ were considered significant.

RESULTS

Effect of catechin on biofilm formation and destruction

To examine the inhibitory effect of EGCg on biofilm formation, *E. coli* (2×10^3 CFU/mL) was incubated with a round PU sheet in a medium containing indicated concentrations (0–0.25 m*M*) of EGCg for 24 h. As shown in Figure 4, bacterial adhesion and growth were dose-dependently inhibited: IC_{50} (50% inhibiting concentration of bacterial adhesion) was estimated to be approximately 0.12 m*M* of EGCg, and the complete inhibition of cell growth was achieved at the concentration of 0.15 m*M*. However, to examine the destruction effect of EGCg on a biofilm (number of adhered *E. coli*: 1.7×10^7 CFU/cm^2) formed by 24-h incubation with medium containing *E. coli*, a biofilm-formed PU sheet was immersed in the PBS containing EGCg at the indicated concentrations (0–50 m*M*) for an addi-

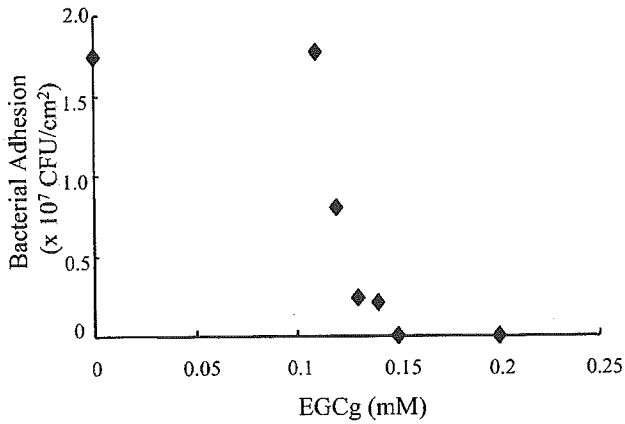


Figure 4. Susceptibility testing of *E. coli* biofilm to EGCg. *E. coli* (2×10^3 CFU/mL) was incubated on a round PU sheet with 0–0.25 m*M* EGCg for 24 h at 37°C under static conditions.

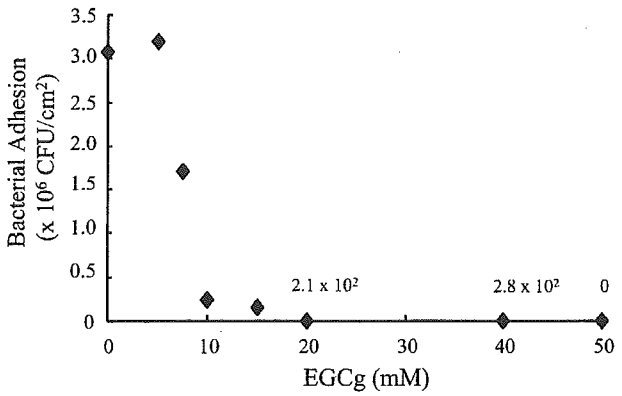


Figure 5. Susceptibility testing of established *E. coli* biofilm to EGCg. *E. coli* (2×10^3 CFU/mL), which was incubated for 24 h in medium without EGCg to form biofilm, was then exposed to 0–50 m*M* EGCg for an additional 24 h at 37°C.

tional 24 h. As shown in Figure 5, a significant decrease in the number of viable *E. coli* cells in biofilm occurred at approximately 6–7 m*M* of EGCg and viable *E. coli* almost disappeared with >20 m*M* of EGCg. The MIC of EGCg, determined by the twofold agar dilution method, was 250–300 μg/mL (Table I).

The growth inhibition and destruction of biofilm was observed under confocal laser scanning microscopy. Live and dead bacteria in biofilms formed by GFP-gene-encoded *E. coli*, which were harvested after 24-h incubation and then immersed in PBS containing EGCg (concentration: 0–50 m*M*), was determined using green fluorescent protein expressed by live *E. coli* cells and red fluorescent staining of dead *E. coli* cells with propidium iodide. As shown in Figure 6, increased concentration of EGCg resulted in decreased green areas but increased red areas. At 20 m*M* of EGCg, most of the *E. coli* were stained red. Irregularly

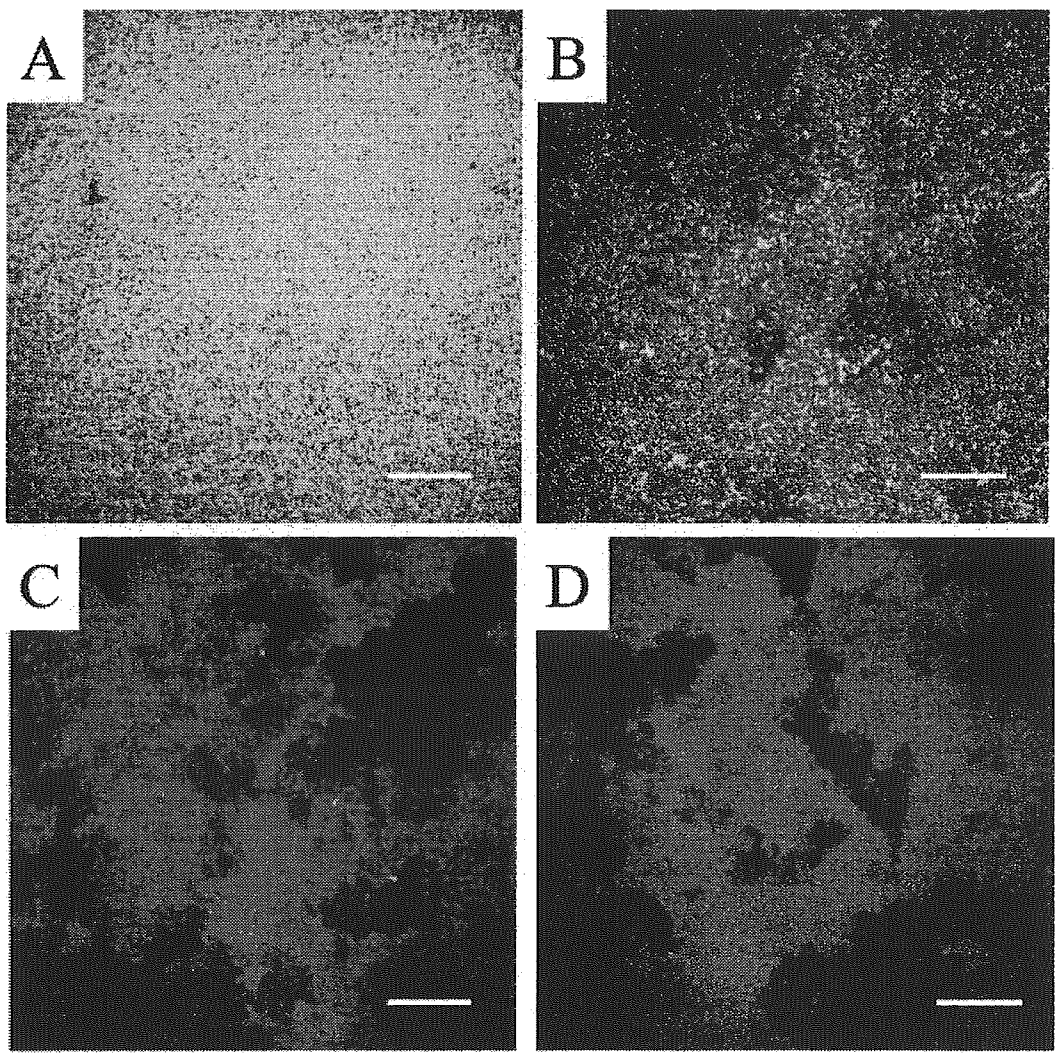


Figure 6. Confocal laser scanning microscopy of biofilm exposed to (A) 0 m*M*, (B) 10 m*M*, (C) 20 m*M*, and (D) 50 m*M* EGCg. Bar: 500 μm.