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MARINE BIOTECHNOLOGY

Surface Attachment Induced Production of Antimicrobial Compounds by Marine Epiphytic Bacteria Using Modified Roller Bottle Cultivation

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Abstract: A modified roller bottle culture method elicited the production of antimicrobial compounds from 2 epibiotic marine bacterial strains, EI-34-6 and II-111-5, isolated from the surface of the marine alga *Palmaria palmata.* These isolates, tentatively identified as *Bacillus* species, were grown as a biofilm on the surface of nutrient glycerol ferric agar (NGFA) and marine Columbia glycerol agar (MCGA) on the inside of a rolling bottle. The biofilm was shown to be stable, and the cells were difficult to remove from the agar surface. The culture supernatant exhibited a different antibiotic spectrum when the strains were grown using the agar roller bottle method compared with shake flask cultures or nonagar roller bottle cultures. These results suggest that biofilm formation is an important factor in the production of antimicrobial compounds by these 2 strains, and roller bottle cultivation also allowed production of these compounds to be increased. The methodology used here has the potential to allow increased production of useful secondary metabolites such as antibiotics from marine epibiotic bacteria.

Key words: surface attachment, antimicrobial compounds, bioreactor, marine epiphytic bacteria, roller bottle cultivation.

INTRODUCTION

Traditional fermentation methods have produced economically important antibiotics. Stirred tank fermentation, which is derived from the laboratory shake flask cultivation method, is most widely used in industrial-scale antibiotic production. Another important method, solid state fermentation (SSF), often applied in food processing, is also used to produce some antimicrobial compounds (Pandey et al., 2000; Robinson et al., 2001). However, newly emerging infectious diseases, reemergent diseases, and multidrug-resistant pathogenic bacterial infections result in a continued need to develop new antibiotics. One of the approaches currently used is to broaden the diversity of extracts used in screening programs. With some compounds derived from marine invertebrates exhibiting relatively unusual structures and biological activities, there is a potential for finding new antibiotics from marine sources (Rinehart et al., 1981). In particular, marine bacteria have recently been identified as a source of new bioactive metabolites (Bernan et al., 1997; Jaruchoktaweechai et al.,

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2000). However, in screening marine bacteria for the production of antibiotic metabolites, fermentations have mainly been carried out under standard culture conditions, especially using planktonic suspension culture, and the design of new culture methods has been neglected (Mearns-Spragg et al., 1998).

In nature most bacteria exist attached to surfaces within biofilms and are inherently different from bacteria existing in the planktonic state (Korber et al., 1995). In marine epibiotic bacteria surface attachment is a significant factor affecting metabolism. Vandevivere and Kirchman (1993) found that the addition of sand to shake flask cultures induced exopolymer synthesis by some surface-isolated bacteria and that exopolymer production by attached cells was greater than that by the same bacteria growing in a planktonic state. Davies and Geesey (1995) compared alginate gene expression in *Pseudomonas aeruginosa* in biofilms and in planktonic cells. The expression of the *algC* gene was increased in biofilm-associated cells compared with planktonic cells. In the sessile state bacteria may release exopolysaccharides to form a thick matrix, which results in the formation of a biofilm (Davies et al., 1993), and cells may alter their morphologies (Dalton et al., 1994; Auerbach et al., 2000) and grow at different rates (Gilbert et al., 1990). Furthermore, Davies et al. (1998) reported that cell-cell signaling could be involved in the differentiation of *Pseudomonas aeruginosa* biofilms, allowing these single-celled organisms to behave as a multicellular organism (Miller and Bassler, 2001). However, very few studies report the effect of surface attachment on production of secondary metabolites.

Screening of marine epibiotic bacteria isolated from the surfaces of marine algae and invertebrates has shown that a high percentage produce antimicrobial metabolites (Lemos et al., 1986; Mearns-Spragg et al., 1997; Boyd et al., 1998, 1999a, 1999b; Burgess et al., 1999). Marine epibiotic bacteria are attracting attention as a potential new source of novel bioactive products (Jensen et al., 1996; James et al., 1996; Imamura et al., 1997). These findings also indicate a relationship between the environmental niche occupied by marine epibiotic bacteria and their metabolism. There have, however, been few studies to show whether allowing these bacteria to grow under certain environmental "niche mimic" conditions—for example, attached to a surface—can induce the production of antimicrobial compounds.

Solid state fermentation allows microorganisms to colonize and form a biofilm on the surface of a solid substrate. However, traditional SSF technology has had limited application because of inadequate diffusion of nutrients and removal of waste (Lonsane et al., 1985; Pandey et al., 2000), which make large-scale production inefficient. Roller bottles have been used for a long time as fermentors in pharmaceutical, biochemical, and medical applications. Tanaka et al. (1983) used a roller bottle bioreactor system for high-density cultivation of plant tissue. This type of system was later used successfully to cultivate *Lithospermum erythrorhyzon* on a 1000-L scale for the production of shikonin, a red dye used in Japanese cosmetics (Tanaka, 1987). Studies of strawberry cell suspension cultures also showed the growth rate of cells to be higher in roller bottles with baffles than in air-lift, shake flask, or stirred-jar bioreactors (Hong et al., 1989). Because this process relies on simple technology, roller bottle culture allows production to be increased simply by increasing the number of bottles. Kunitake and colleagues (1997) reported a fully automated roller bottle production facility for factory use. In addition, roller bottle culture can provide unique growth conditions when cultivated cells are anchored or attached directly or indirectly to the inner wall of a roller bottle, in which case those cells can form a biofilm and are periodically in contact with both the gas and the liquid phase, conditions similar to the continuous wetting and air exposure experienced by bacteria growing on intertidal seaweeds. However, it has proved difficult to anchor most kinds of plant, animal, and bacterial cells to the inner wall of roller bottles using traditional methods (Muzzio et al., 1999, Unger et al., 2000). Therefore, it was necessary to find a simple but effective method for anchoring bacterial cells, directly or indirectly, to the inner wall of roller bottles.

During preliminary studies it was found that the epibiotic marine bacterial strains, EI-34-6 and II-111-5, isolated from the surface of the seaweed *Palmaria palmata,* remained attached to the agar surface once grown on agar medium, even when mixed or washed with the corresponding broth medium. This article describes the production of antibiotics by these 2 strains using a novel roller bottle cultivation method that relies on an inner coating of agar and its effect on antibiotic production.

Materials and Methods

Bacterial Strains

The production of antibacterial compounds by 2 epiphytic marine bacterial strains, II-111-5 and EI-34-6, isolated

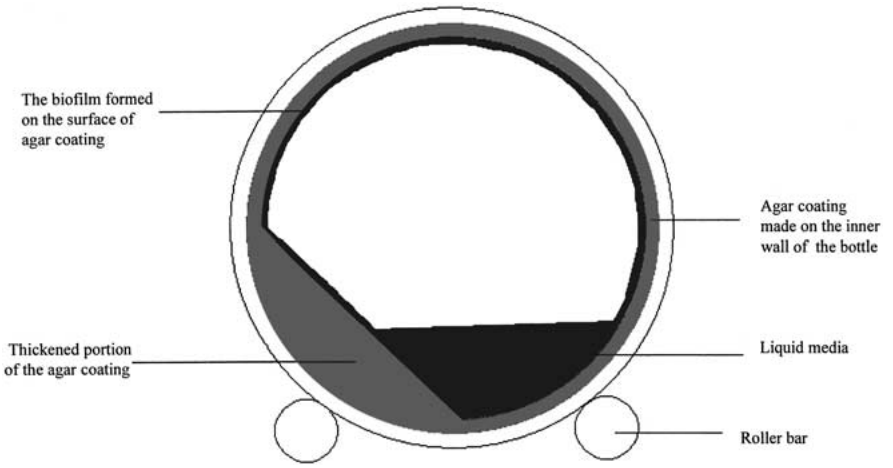

Figure 1. Roller bottle cross section. A layer of NGFA or MCGA coating was made on the inner wall of a 500-ml Duran bottle with one fraction thickened. EI-34-6 or II-111-5 was spread on the surface of the agar coating by a swab and cultivated statically for different times. Then 50 ml of the corresponding liquid medium, NGFB or MCGB, was added and the bottle was rolled at 1 rpm.

from the surface of *Palmaria palmata,* was investigated. Antibacterial activity was tested against a range of bacteria, which were a kind gift from Professor Sebastian Amyes, Department of Medical Microbiology, University of Edinburgh: 3 strains of methicillin-resistant *Staphylococcus aureus* (MRSA), 4, 9551, and 14986; 2 strains of vancomycin-resistant *Enterococcus faecium*, VRE788 and VRE6155; and 2 strains of vancomycin-resistant *Enterococcus faecalis*, VRE1349 and VRE8000.

Preparation of Surface-Attached Roller Bottle Culture

An agar coating was deposited on the inside wall of a Duran bottle with one part deliberately thickened (Figure 1). Two kinds of agar media were prepared: one, termed marine Columbia glycerol agar (MCGA), was mixture of marine agar (Difco) and Columbia agar (Oxoid) in a ratio of 3:2 (wt/wt), with glycerol added to a final concentration of 1% (vol/vol); the other, termed nutrient glycerol ferric agar (NGFA), consisted of nutrient agar with ferric chloride and glycerol added to concentrations of 0.2 g/L and 1% (vol/vol), respectively. We used 100 ml of the MCGA or the NGFA to coat the inside wall of a 500-ml Duran bottle. The agar coating could be achieved during solidification of the agar after autoclaving by rolling on ice. The MCGA or the NGFA coating was allowed to solidify at room temperature for 24 hours, and 40 μl of a 2-day culture of II-111-5 or EI-34-6 grown at 28°C in marine broth was spread on the MCGA or NGFA coating using a swab. The Duran bottles were incubated under static condition at 28°C for 1 to 7 days to initiate an agar surface culture (for biofilm formation), after which time 50 ml of the corresponding liquid medium, marine Columbia glycerol broth (MCGB) or nutrient glycerol ferric broth (NGFB), was added. The bottles were then rolled horizontally on a Profiler Roller M1241-6001 (New Brunswick Scientific) at 1 rpm at 28°C, allowing the biofilm on the agar surface to be periodically exposed to the air, and submerged in the liquid media. At defined time intervals 1.5-ml samples of the liquid media in the roller bottle cultures were taken to test for antibacterial activity.

Two types of controls were used for each strain. Control a containing 100 ml of the NGFB (control E-a) or the MGCB (control II-a) without the corresponding agar coating was inoculated with 40 μl of EI-34-6 or II-111-5, respectively, as mentioned above. Control b was a 4-day surface culture on the NGFA (control E-b) or the MCGA (control II-b) coating, as mentioned above. In the case of control b, the coating on which the bacterial biofilm had formed was then dislodged from the bottle wall using a sterile spatula and mixed with 50 ml of the corresponding broth so that the bacterial biofilms with agar medium were submerged in the liquid media during the remaining cultivation time.

Shaken Flask Culture

Flasks containing 25 ml of MCGB or NGFB were inoculated with 10 μl of a 2-day culture of II-111-5 or EI-34-6, respectively. These flasks were incubated with shaking at 200 rpm at 28°C for 4, 6, and 12 days before the medium was removed and tested for antibacterial activity.

Determination of Antibacterial Activity

At defined time intervals 1.5-ml samples of the liquid media in the roller bottle cultures were taken to test for antibacterial activity. The time points when samples were

Table 1. Growth Time of Biofilms on Agar Coating Before Addition of Liquid Media

Bacteria	Culture	Pregrowth time of biofilms
EI-34-6	E-a	No biofilm
	E-b	4 days†
	E-0	2 hours
	E-1	1 day
	E-2	2 days
	E-3	3 days
	E-4	4 days
	E-5	5 days
	E-6*	6 days
	E-7*	7 days
II-111-5	II-a	No biofilm
	II-b	4 days†
	II-0	2 hours
	II-1	1 day
	II-2	2 days
	II-3	3 days
	II-4	4 days
	II-5	5 days
	II-6	6 days
	II-7	7 days

*Agar coating disintegrated into the added broth medium during rolling cultivation.
†Agar coating was dislodged before the addition of liquid medium.

removed from each bottle are described in Table 1. Antibacterial activity was tested using the paper disk assay (Mearns-Spragg et al., 1997). Each sample was centrifuged at 13,000 rpm for 10 minutes to pellet the bacterial cells. The supernatant, 2 × 30 µl, was used to saturate antibiotic assay disks (6-mm, Whatman), with a period of drying between each application. Test bacteria were swabbed onto Columbia agar plates. The disks were then placed onto the agar surfaces, and the plates were incubated at 37°C for 12 hours. The diameters of any inhibition zones that had formed around the paper disks were then measured.

Determination of Bacterial Cell Concentration in Liquid Phase

Bacterial cell concentrations, in the liquid phase, were monitored at various times (Table 1) by recording the optical density at 660 nm (OD_{660}) using a PC-controlled Shimadzu UV-1601 UV-visible spectrophotometer. Sterile MCGB or NGFB was used as a blank.

Taxonomic Study of EI-34-6 and II-111-5

Several biochemical tests were used to identify the strains. Gram stain, Schaeffer-Fulton spore stain, and the oxidase and catalase reactions were carried out as detailed by Collins et al. (1995). Starch, casein, and gelatin hydrolysis, propionate utilization, anaerobic growth, and Voges-Proskauer reactions were carried out as detailed by Parry et al. (1983).

Determination of Sporulation Level

Sporulation level of EI-34-6 and II-111-5 was measured using the Schaeffer-Fulton spore stain method. Bacteria on agar coating were removed from the surface using a loop, then diluted and dispersed in 1 ml of NGFB. Also, 50 µl of liquid culture was diluted in the same way. A loopful of diluted sample was stained on a slide. Spores (blue-green) and vegetative cells (red) were counted under the microscope. The percentage sporulation (ratio of spore number to total cell number) was determined by the average of 5 random fields of vision.

Results

Growth Characteristics of Two Strains of Marine *Bacillus* in Roller Bottle Culture with Inner Agar Coating

Strain EI-34-6 with NGFA Coating

In the control culture without the NGFA coating, EI-34-6 grew in liquid NGFB and there was no obvious biofilm on the glass wall of the roller bottle, nor was the production of pigment observed. With the NGFA coating, EI-34-6 showed very different growth characteristics on the coating surface, and these varied according to when the rolling culture was initiated. When the liquid medium NGFB was added to the roller bottle on the same day, 2 hours after EI-34-6 was spread on the surface of the agar coating NGFA (bottle E-0), no biofilm or pigment was observed over the next 7 days. If the NGFB was added on the 2nd day (bottle E-1), after the bacteria had been allowed to grow on the surface of the inner NGFA coating for 1 day, a thin biofilm was observed on the surface of the coating.

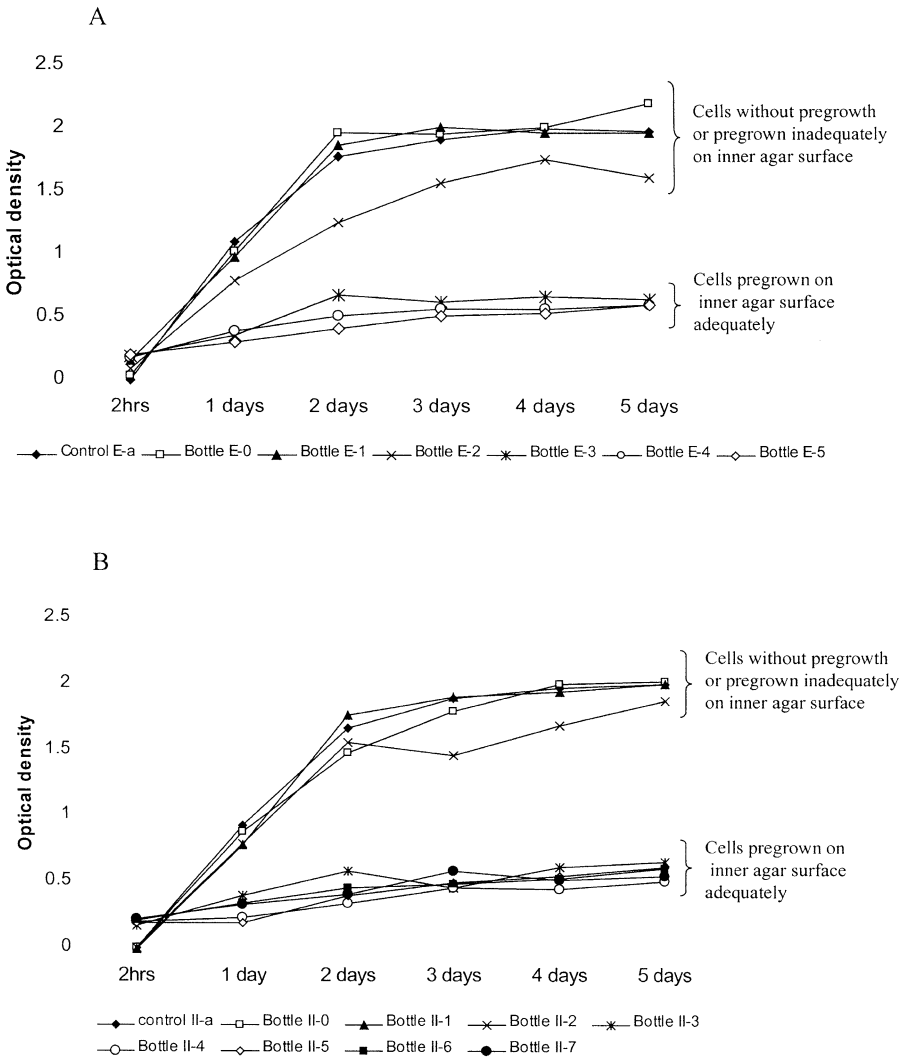

Figure 2. The optical density of the liquid medium added after pregrowth of the biofilms with rolling time. **A:** EI-34-6 cultures (NGFB). **B:** II-111-5 cultures (MCGB). Refer to Table 1 for definition of labels. The OD values of bottles E-a, E-0, E-1, and E-2 showed apparent increases with time, which suggests that bacteria grew in the NGFB. Bottle E-a showed changes similar to those in E-0 and E-1, suggesting the same growth patterns of bacteria in these 3 bottles. Bottles E-3, E-4, and E-5 showed no obvious increase of OD with rolling time, suggesting that not many bacteria were growing in the liquid medium. II-111-5 cultures showed growth patterns similar to those of EI-34-6.

The growth of bacteria in the liquid medium was also obvious during the following cultivation period. However, bacteria on the coating did not produce red pigment when the liquid broth was added, or during the subsequent 6 days of rolling cultivation. Bottle E-3 contained a 3-day-old biofilm on the inner agar surface and showed pigment production. Upon addition of the liquid medium, the red pigment did not dissolve into the liquid medium, and the NGFB retained its original yellow color during the whole cultivation process. In cultures E-0, E-1, and E-2, the bacteria growing in the liquid broth could be clearly seen; however, the cell density in the NGFB from E-2 was lower than that from E-1 during the same period of rolling cultivation. Growth of the bacteria in the roller bottles to which the NGFB was added on the 4th day (E-3) or later (E-4, E-5, E-6, and E-7) showed similar growth characteristics, with bacterial cells forming a stable biofilm on the surface of the NGFA coating and with only a small number of cells present in the liquid medium (Figure 2A). The production of the red pigment was also obvious in the biofilm.

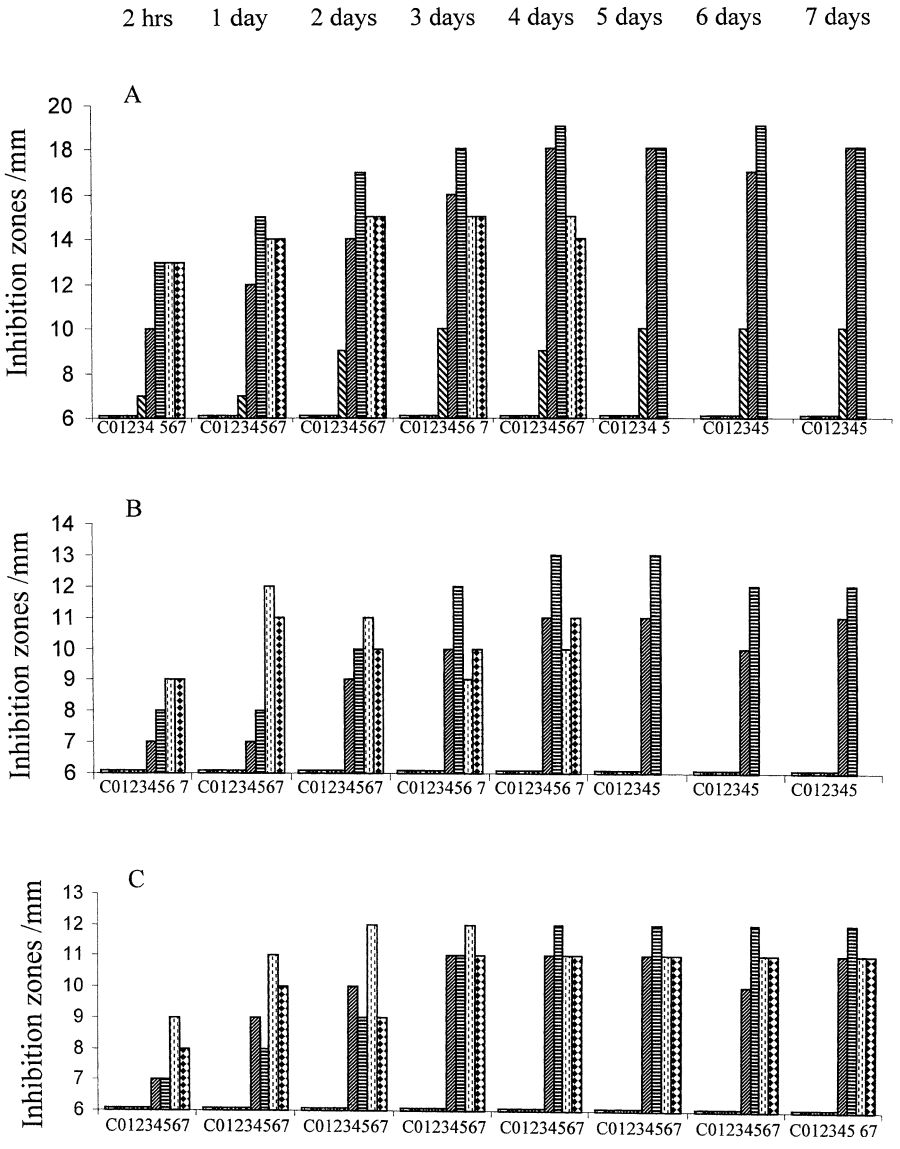

Figure 3. Comparison of the antibiotic activity in liquid media from bottle cultures with different rolling times. **A:** Activity of EI-34-6 cultures against MRSA 9551. **B:** Activity of EI-34-6 cultures against VRE 6155. **C:** Activity of II-111-5 cultures against MRSA 4. The times that EI-34-6 and II-111-5 were allowed to grow on the solid agar surface before addition of the liquid media were varied. Bar C is control E-a (A and B) or II-a (C), having no agar coating; bars 0 to 7, bottle E-0 to E-7 (A and B) or II-0 to II-7 (C). Aliquots of the liquid medium were removed from each bottle and tested for antibiotic activity. Detectable activity was found in media from E-3 (A and B) or II-3 (C), with E-4 and E-5 showing the highest activities.

Strain II-111-5 with MCGA Coating

Apart from the absence of pigment production, II-111-5 showed growth characteristics similar to those of EI-34-6 (Figure 2B). A biofilm was formed on the inner MCGA coating on the 3rd day after inoculation, and similarly only a small amount of cells were present in the liquid medium once the biofilm had formed.

Comparison of Antibiotic Activity in Different Cultures

EI-34-6

Shake flask cultures showed no antibiotic activity. Rolling culture suspensions of the control E-a (without the NGFA coating) and bottles E-0, E-1 and E-2 (without

obvious biofilm formation) showed very little antibiotic activity, as well as a narrow activity spectrum; this was quite different from the cultures in bottles E-3, E-4, E-5, E-6, and E-7, which began with mature biofilms on the inside of the bottle. The spectra of antibiotic activity in the culture suspensions to which the NGFB was added on the 4th day (bottle E-3) or later (bottles E-4 to E-7) were similar, except for the magnitude of the activity. Taking the activity of EI-34-6 cultures against MRSA 9551 (Figure 3A) and against VRE 6155 (B), for example, the results showed that the antibiotic activity of cell suspensions from the rolling culture were highest 4 days after addition of the liquid medium. The liquid removed from bottles in which the biofilm had been allowed to establish itself for 3 or 4 days prior to the addition of the liquid medium displayed greater activity. Activity against other test strains exhibited similar patterns. Control E-b, in which there was surface cultivation on the NGFA coating followed by a totally submerged suspension culture (see "Materials and Methods"), showed few changes in intensity of antibiotic activity during the rolling cultivation process compared with bottle E-4 (Figure 4A).

II-111-5

Shake flask cultures of II-111-5 also showed no activity. Rolling culture suspensions of the control II-a (without the MCGA coating) and bottles II-1 and II-2 (without obvious biofilm formation) showed little antibiotic activity, but suspensions from the cultures in bottles II-3 to II-7 showed obvious activity against 3 MRSA strains. As an

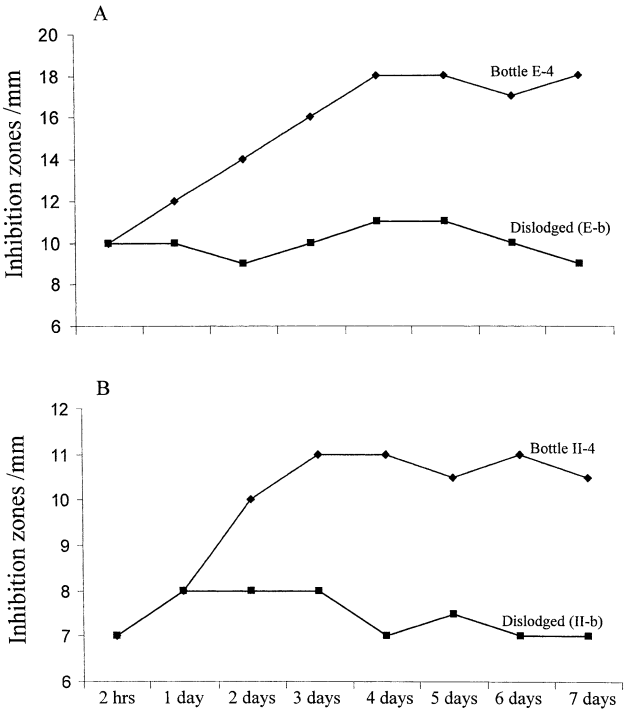

Figure 4. Difference in antibiotic activity between coating-dislodged culture and E-4 **(A)** or II-4 **(B)**. Two bottles of EI-34-6 or II-111-5 were cultivated on the surface of the agar coating for 4 days. The agar coating in one bottle was then dislodged before the addition of the liquid medium, so that the bacteria, with the dislodged agar coating, were submerged in the liquid medium during the whole rotation period. The other bottle contained a normal roller bottle culture, as with E-4 or II-4. The activity of E-4 and II-4 against the MRSA 9551 increased with rolling time and reached its highest point 4 or 5 days after the addition of the liquid media, but E-b and II-b showed no obvious change in the activity over time.

Table 2. Taxonomic Study of EI-34-6 and II-111-5 and Two Representative *Bacillus* Species

Test	EI-34-6	II-111-5	*B. subtilis*	*B. licheniformis*
Gram staining	+	+	+	+
Spores	+	+	+	+
Growth in 7% NaCl	+	+	+	+
Catalase	+	+	+	+
Strong adherence to agar surface	+	−	−	+
Anaerobic growth	+	−	−	+
Voges-Proskauer	+	+	+	+
Citrate utilization	+	+	+	+
Nitrate reduction	+	+	+	+
Starch hydrolysis	+	+	+	+
Propionate reaction	+	−	−	+
Casein hydrolysis	+	+	+	+
Gelatin hydrolysis	+	+	+	+
Oxidase	+	+	+	+

example, the II-111-5 cultures showed greatest activity against MRSA 4 (Figure 3C) 3 or 4 days after the addition of the MCGB medium, when the biofilm had been allowed to grow for 3 or 4 days. Control II-b in which there was surface cultivation on the MCGA coating followed by a totally submerged suspension culture, also showed few changes in intensity of antibiotic activity during the rolling cultivation process compared with bottle II-4 (Figure 4B).

Sporulation Study

Staining by the Schaeffer-Fulton method revealed approximately 60% sporulation in all cultures (shaken flasks, biofilm, and rolling culture with or without coating) when cultivation was carried out for 4 days or more. Spore level in the biofilm, however, decreased to approximately 20% within 2 days after fresh broth medium was added and then increased to the levels present before addition of biofilm with 4-day pregrowth.

Identification of EI-34-6 and II-111-5

Tentatively, EI-34-6 was identified as *Bacillus licheniformis* and II-111-5 as *Bacillus subtilis* according to morphological and biochemical characteristics (Table 2). Preliminary 16S ribosomal DNA sequence analysis also suggested that both strains were species of *Bacillus* (data not shown).

Discussion

In suspension cultures, either in a shake flask or in standard roller bottle cultivation, *Bacillus* strains EI-34-6 and II-111-5 did not form obvious biofilms on the glass surface. The glass surface does not appear to be able to mimic the ecological niche of these strains, i.e., seaweed surface. *Bacillus licheniformis*, strain EI-34-6, characteristically forms colonies that are usually strongly attached to the agar surface; thus the agar-coated roller bottle method takes advantage of this phenomenon, facilitating the formation of a strongly bound biofilm of EI-34-6. Although the liquid medium NGFB provided sufficient nutrients, it appeared that the cells preferentially adhered to the surface of the NGFA coating. Strain II-111-5 was identified as a strain of *B. subtilis*, and this species does not usually adhere to agar as strongly as *B. licheniformis*. However, this marine isolate showed a similar phenomenon, preferring growth in the biofilm when in the presence of a liquid nutrient source.

This study also highlights another important phenomenon. Although the medium provided the same nutrients, cells attached to the agar surface produced antibacterial compounds that planktonic cells could not. Rolling cultivation for 2 hours after the addition of liquid medium appeared to result in the dissolution of metabolites that were produced by attached cells. Thus the activities of "2 hours" show that antibacterial compounds were produced during biofilm growth. When there was detectable activity (bottles E-3 to E-7 or II-3 to II-7), biofilm formation was always observed. Bottles E-0, E-1, II-0, and II-1 and controls E-a and II-a had no biofilm, and the liquid media associated with these cultures had no detectable activity.

Antibiotic production by many *Bacillus* strains is accompanied by sporulation (Msadek, 1999). However, although sporulation levels were similar in all the cultures, this did not correlate with antibiotic activity, indicating that some other factors may play a role in inducing the production of the antibacterial compounds. It has been reported that many bacterial strains upon attaching to a surface produce exopolysaccharides or exopolypeptides (Davies et al., 1993, 1998; Davies and Geesey, 1995; Allison et al., 1998). In addition, it has been postulated that exopolysaccharides could mediate the attachment of the bacteria to the surface and induce metabolic changes (Allison and Sutherland, 1987; Pratt and Kolter, 1999), suggesting production of different metabolites under attached growth conditions.

Recent studies have shown that many bacteria, including *B. subtilis*, produce signal molecules that are used by the cells to monitor cell density, a phenomenon termed quorum sensing, which also controls cell metabolism (Swift et al., 1996; Surette and Bassler, 1998; Msadek, 1999). Cells in biofilms usually grow at higher cell densities than in liquid cultures, and quorum-sensing mechanisms could affect the production of secondary metabolites by these cells. However, most quorum-sensing studies use suspension cultures, in which signal molecules secreted into the liquid culture induce a response in the bacterial population (Dunny and Winans, 1999). Since both EI-34-6 and II-111-5 were able to reach a cell density as high as 10^9 cfu/ml in shake flask cultures without any measurable production of antibiotics, other induction mechanisms may be regulating antibiotic production in this system. In fact, most cells within a biofilm are in immediate contact with their neighbors, unlike planktonic growth, so further investigation is needed to find out whether unique signal systems exist for

communication between bacterial cells in such close proximity, or whether sensing of the physical attachment itself can trigger changes in expression of genes associated with antibiotic synthesis.

When NGFB or MCGB was added at different times after inoculation of the agar surface, followed by rolling cultivation, the increased antibiotic activity observed in the first few days indicated that the rolling cultivation method could elicit production of antibiotic compounds by EI-34-6 and II-111-5. When EI-34-6 was cultivated on the agar surface for 3 days (bottle E-4) followed by the addition of the liquid medium and rolling cultivation, antibiotic activity of the liquid medium against MRSA 9551 increased. Activity was highest after 3 to 4 days (Figure 3A). The activity of EI-34-6 culture against the other test strains showed a similar change, as did II-111-5 culture against 3 MRSA strains. However, if the NGFA or the MCGA coating was dislodged before the addition of the corresponding liquid medium, so that EI-34-6 or II-111-5 was submerged in the liquid during the whole period of rolling cultivation, no obvious change of the antibiotic activity with rolling time was observed (Figure 4). This suggests that when EI-34-6 or II-111-5 formed a biofilm on the coating surface and started to produce antibiotic compounds, periodic exposure to the liquid medium and to air could have an important effect on the biofilm, allowing the continued production of the antibiotic compounds. This is the first report comparing metabolite production of anchored cells under different cultivation methods. The mechanism by which periodic exposure to liquid medium and to air affects antibiotic production is not yet clear. However, these conditions mimic the ecological niche of the microbes on intertidal seaweed, and we have therefore termed this type of reactor a ''niche mimic bioreactor.''

Roller bottle bioreactors are widely used in industry. Typical roller bottle bioreactors have unique flow dynamics. Muzzio and colleagues (1999) employed a computer-simulated particle-settling program to simulate cell flow in a roller bottle culture. A substantial fraction of the cells in a roller bottle could actually remain trapped indefinitely in recirculating trajectories within the bottle, never reaching the bottle wall. Practically, the flow and mixing dynamics of roller bottle bioreactors, in the production of certain vaccines, has been found to decrease the possibility of the infected cells attaching to the host cells anchored on the bottle walls (Elliot, 1990). It was also observed in our experiment that if EI-34-6 or II-111-5 was not forced to form a biofilm on the surface of the NGFA or the MCGA coating by SSF in advance, the cells would not adhere to the bottle walls themselves to form a biofilm from the suspension culture, even if the NGFA or the MCGA coating was provided. Thus it would be difficult to investigate the effects of periodic exposure to a liquid medium and then to air on the production of antibacterial metabolites.

In addition, for the cultivation of animal or plant cells at high cell density, general roller bottle bioreactors have an intrinsic problem with the supply of oxygen to the growing cells (Tanaka, 1987). Unger and colleagues (2000) investigated flow and fluid-mixing patterns of the general roller bottle bioreactor and also found that the fluid mixing was greatly hindered by the lack of efficient axial mixing. However, if the roller bottle was installed with baffles, the oxygen transfer rate could be greatly improved (Hong et al., 1989; Tanaka, 1987). In this study the thickened fraction of the agar coating was designed to increase oxygen supply by functioning as a baffle.

Our results indicate that where bacteria produce important metabolites under surface attached conditions the modified roller bottle cultivation method described here can be used to increase production of important metabolites. It may also prove useful for the design of new antibiotic screening programs based on the use of SSF of surface-associated bacterial isolates. In addition, we expect eco-process engineering, the introduction of ecologically relevant processes into bioprocess engineering, to increase in the future.

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