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Comparison of colony lift with direct spotting methods of blot preparation on the effect of colony hybridization in the detection of environmental organisms

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Nucleic acid probes are used on site to detect or to identify individual microbial cells without cultivation. This molecular technique can avoid some limitations of traditional identification methods including time consuming and imprecise. This study examined the factors affecting colony hybridization and compared the effectiveness of membrane prepared by colony lifting with direct spotting procedures using the universal probe Eub 338. The results of hybridization varied depending on the type of colony morphology. For dry and rough colonies, colony hybridization was not suitable for detecting *Acinetobacter* sp. (CK2A, CK2B), *Alcaligenes* sp. (TH11B), *Xanthomonas* sp. (TH7B), *Arthrobacter globiformis* (CCRC 10598) and *Microbacterium* sp. (CCRC 11036). Colonies of *Acinetobacter* sp. (CCRC 15425) and *Alcaligenes* spp. (CCRC 10828, H) on agar and membrane were thick and raised, and their detection signals of hybridization were diffused or blank. Colonies of *Alcaligenes* sp. (CM7A, ANV2) and *Acinetobacter* sp. (ANV8) isolated from the sludge of biological processes treating ABS wastewater were flat and smooth, and their hybridization signals were clear. For those strains suitable for colony hybridization, the colony blots prepared by colony lift and direct spotting procedures gave the same sensitivity for colony hybridization.

Key words: Colony hybridization, colony lift, direct spotting method, nucleic acid probes

The analysis of the structure and function of natural microbial communities has traditionally relied on cultural, physiological and biochemical techniques that are frequently time-consuming and sometimes imprecise [1]. Molecular technique which uses nucleic acid probes has been developed and is now commonly applied in the detection of food-borne bacteria. The technique requires a relatively short period of time compared to laboratory methods and has a high degree of precision [2,3]. Using the ST-P probe, Moseley *et al* [4] detected enterotoxigenic *Escherichia coli* with a specificity of 71% (12 of 17 isolates). A synthetic oligonucleotide DNA probe was also found to be highly specific (100 %) for the identification of *Vibrio parahaemolyticus* [5,6].

Various preparation formats can be used for the detection of specific hybridization, for example on solid support, in solution or *in situ*. Colony hybridization is perhaps the simplest application of nucleic acid hybridization and the easiest to integrate with conventional environmental microbiological sampling and analysis [7]. This technique was originally developed to facilitate the isolation of specific recombinant DNA clones in *E. coli* [4]. It requires 2 to 2.5 weeks to identify an isolate from the environmental sample [1] while only 2 to 3 days are required using colony hybridization [8]. There are three methods for the preparation of filter membranes used in solid hybridization: the direct spotting method [4], colony lift [8] and cell filtration [2]. The direct spotting method was developed by Moseley *et al* [4] and is performed by placing a sterile membrane on the surface of the agar plate and directly inoculating or spreading the bacterial cells. Overgrown bacterial colonies usually result in an interfering background [9]. The colony lift method is done by lifting the bacterial colonies from the agarose plate onto the filter and then processing for hybridization [10]. Membranes for colony hybridization could be prepared by filtration of bacterial cell culture

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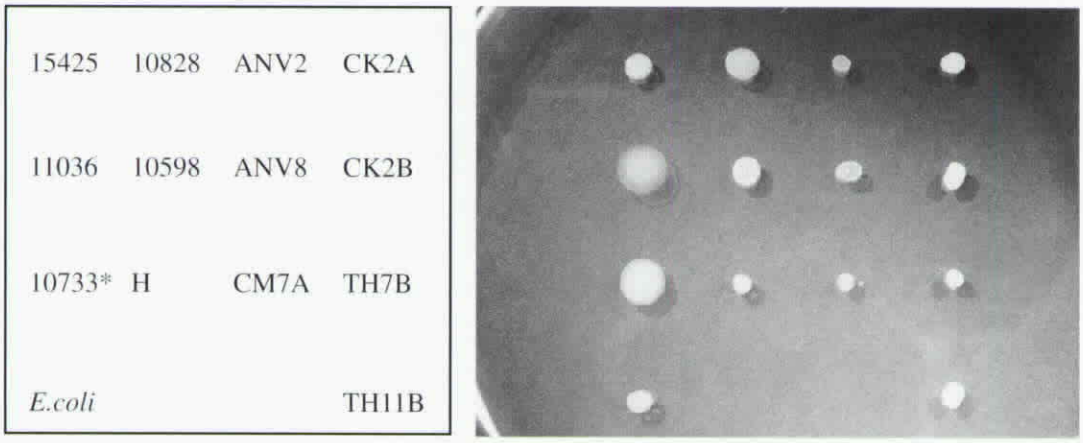

Fig. 1. Colony morphologies of tested strains on TSA. On the left panel indicated the position of strain inoculated, *control strain.

with the Bio-dot apparatus [11], however, cellular loss occurred when the apparatus was not tightly sealed during filtration.

It was confirmed that heterotrophic nitrification bacteria exist in wastewater treatment processes [12]; however, a lengthy time period was required to isolate, purify, identify and enumerate these microorganisms. The purpose of this study was to use the nucleic acid probe techniques to detect heterotrophic nitrification bacteria in biological wastewater. The universal probe Eub 338 [13], which can probe to all eubacteria, was used to assess the feasibility of using colony hybridization to detect heterotrophic nitrification bacteria. The colony characteristics of heterotrophic nitrifiers and the effect of preparation of filter membrane on colony hybridization were investigated.

Materials and Methods

The bacterial strains used in this study are listed in table 1. Tryptic soy broth (TSB, Difco Laboratory, MI, USA) was used for culturing all strains, and bacterial strains were incubated at 37 °C. Most reagents were purchased from Boehringer Mannheim (Germany). CSPD® (Disodium 3-(4-methoxyspiro [1, 2-dioxetane-3, 2'-(5'-chloro)-tricyclo [3,3,1,1^{3,7}] decan]-4-yl) phenyl phosphate) was purchased from Tropix (MA, USA). Eub 338 probe [13] was commercially synthesized and labeled with digoxigenin according to "The Dig System User's Guide for Filter Hybridization" [10]. The filter membrane was positively charged Nylon-66 (Boehringer Mannheim) and was prepared with the colony lift [8] or the direct spotting method [4]. Colony hybridization was carried out according to standard procedure [10]. CSPD® was used for the detection of hybridization.

Results and Discussion

Effect of characteristics of colony morphology on colony hybridization

The dot blotting or direct spotting method for intact cells is suitable for screening large numbers of samples [7], however it is difficult to find a proper medium and temperature for all tested strains to grow within a short period of time. Among the 13 strains tested in this study, we found that all strains could grow on tryptic soy agar (TSA). Since most isolates were isolated at 28 °C [12], it took 24 h to 36 h to form colonies of 2 mm to 3 mm at 28 °C, but it took only 12 h to 24 h for most strains to form reasonable size of colonies at 37 °C (Table 2). Therefore, tested strains were incubated at 37 °C for both the colony lift and direct spotting methods. Only

Table 1. Bacterial strains used in this study and their sources

Strains	Sources of isolates
Alcaligenes latus (ANV2)	Fluidized bed[b]
Acinetobacter gonospecies (ANV8)	Fluidized bed[b]
Alcaligenes latus (CM7A)	Activated sludge[b]
Acinetobacter gonospecies (CK2A)	Fluidized bed[b]
Acinetobacter radioresistens (CK2B)	Fluidized bed[b]
Xanthomonas oryzae pv oryzae E (TH7B	SBBR[b]
Alcaligenes latus (TH11B)	SBBR [b]
Acinetobacter radioresistens (CCRC[a] 15425)	
Alcaligenes faecalis (CCRC 10828)	
Arthrobacter globiformis (CCRC 10598)	
Microbacterium sp. (CCRC 11036)	
Alcaligenes xylosoxidans (H[c])	SBBR[b]
Escherichia coli (CCRC 13082)	

[a]Culture Collection and Research Center (CCRC, FIRDI)
[b]reference 12
[c]reference 14

Table 2. Effect of incubation temperature on the formation of colony with 2-3 mm in diameter

Strain	Incubation time (h)	
	28 °C	37 °C
ANV2	36	24
ANV8	36	24
CM7A	36	24
TH7B	24	20
TH11B	24	12
CK2A	24	20
CK2B	24	20
15425	12	10
10828	12	10
11036	6	6
10598	20	36
H	12	10
E. coli	6	6

seven out of 13 tested strains showed a positive result of colony hybridization with both colony lift and direct spotting techniques. Table 3 shows the characteristics of the tested strains showing negative results of hybridization. The colonies of strains CK2A, CK2B and TH7B appeared to be dry and wrinkled (Fig. 1). The negative results for these three strains might have been due to failure to transfer enough cells to the blot by colony lift or to incomplete reaction for lysis, denaturation and fixation of cells to the membrane [15]. Although TH11B also belong to the *Alcaligenes latus* strain, as does CM7A (Table 1), it showed a negative result in colony hybridization. TH11B and CM7A were isolated from different sludge samples [12], hence their stain characteristics might be different. This result is similar to that described by Lee *et al* [16] who used VP5 probe for the detection of *V. parahaemolyticus*, and found that six out of 95 tested strains had negative results. For strain 11036, the existence of a thin membrane on the colony might have interfered with the processes of denaturation, fixation and washing [9], and result in a negative signal. At 37 °C it took about

Table 3. Colony characteristics of tested strains showing negative results of colony hybridization[a]

Strains	Characteristics of colony
CK 2A	Dry and wrinkle
CK 2B	Dry and wrinkle
TH7B	Dry and wrinkle
TH11B	Shining
11036	Covered by thin membrane
10598	No colony formed

[a]Strains incubated at 37 °C for 24 h on TSA

Table 4. Effect of membrane preparation on the colony hybridization[a]

Strains	Positive rate (%)	
	Colony lift	Direct spotting
E. coli	64 (16/25)[b]	56 (5/9)
10828	74 (29/39)	78 (7/9)
15424	37 (13/35)	57 (4/7)
ANV2	50 (5/10)	80 (4/5)
ANV8	50 (5/50)	80 (4/5)
CM7A	57 (4/7)	75 (3/4)
H	84 (26/31)	100 (9/9)
Mean	59	75
P (α = 0.05)	0.08	

[a]Positively charged membrane was used in this experiment.
[b]Numbers in the parentheses are number of positive colonies divided by total number of colonies tested.

36 h for strain 10598 to form a colony of 2 mm to 3 mm (Table 2), therefore, when incubated for 16 h to 28 h, the colony of 10598 was too small to obtain a signal of colony hybridization.

Strains with raised, smooth, and sticky colonies or smooth and flat colonies (Fig. 1) had clear signals of colony hybridization. Hence it was not feasible to detect strains having small colonies, dry colonies or colonies covered with thin membrane by colony hybridization.

Effect of membrane preparation on the colony hybridization

Table 4 shows the effect of filter membrane preparation on colony hybridization. The tested strains were those suitable for colony hybridization (Fig. 1). Blots prepared by colony lift and direct spotting method had a positive result in 37% to 84% and 56% to 100% of tests, respectively. Although the average of positive result for the colony lift method (59%) was lower than that of the direct spotting method (75%), analysis using t test indicated that the difference between these two methods was not significant. The blots prepared by colony lift and direct spotting procedures had the same sensitivity for colony hybridization. The results of this study suggest that the direct spotting method is a simple, rapid, and better procedure for filter hybridization.

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