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NOTE

Freeze-drying of *Flavobacterium columnare*, *Flavobacterium psychrophilum* and *Flexibacter maritimus*

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ABSTRACT: Freeze-drying of bacteria requires the use of a cryoprotective agent. Skim milk, polyvinylpyrrolidone, dextran, sodium glutamate, sucrose, horse or calf serum are all routinely used. The effects of 7 suspending media, each containing 1 of these cryoprotective additives, were studied on 3 strains each of 3 fish pathogenic gliding bacteria (*Flavobacterium columnare*, *Flavobacterium psychrophilum* and *Flexibacter maritimus*). The viability of preserved bacteria was evaluated by colony enumeration on Anacker and Ordal medium (*Fa. columnare* and *Fa. psychrophilum*) and on Difco marine medium 2216E (*Fx. maritimus*), 48 and 72 h after inoculation. The data show the 3 bacterial species to be optimally preserved in suspending media containing Difco Bacto Brucella Broth (2/3) supplemented with either horse or foetal calf serum (1/3).

KEY WORDS: Freeze-drying · Cryoprotection · *Flavobacterium* · *Flexibacter* · Fish pathogen

Three species of gliding bacteria are well recognized fish pathogens. *Flavobacterium columnare* (= *Flexibacter columnaris* = *Cytophaga columnaris*; Bernardet et al. 1996) has been reported as a pathogen for many freshwater fish species throughout the world (Austin & Austin 1987). *Flavobacterium psychrophilum* (= *Flexibacter psychrophilus* = *Cytophaga psychrophila*; Bernardet et al. 1996) is responsible for high mortality in salmonids, particularly in coho salmon *Oncorhynchus kisutch* in the USA and Canada (Holt et al. 1993) and in rainbow trout *Oncorhynchus mykiss* in Australia and several European countries (Schmidtke & Carson 1995). *Flexibacter maritimus* causes fish disease in sea farms in Japan (Wakabayashi et al. 1986), Scotland (Bernardet et al. 1990), Spain (Alsina & Blanch 1993), and France (Bernardet et al. 1994). The laboratories studying these bacterial species, as well as the culture collections, maintain numerous bacterial strains using different preservation methods. For long-term preservation, suspension in casitone 1% at −80°C, ultra-freezing in liquid nitrogen (−196°C), and freeze-drying are currently employed. The freeze-drying technique is often preferred because of its convenience for the storage and shipping of samples. The efficiency of this long-term preservation procedure depends, among other parameters, on the suspending method and on the cryoprotective agent included in the suspending medium which must be adapted to the species lyophilized. Studies carried out to compare 7 suspending media for freeze-drying of *Fa. columnare*, *Fa. psychrophilum*, and *Fx. maritimus* strains are reported here.

Materials and methods. The 9 bacterial strains included in this study are listed in Table 1. The *Flavobacterium columnare* and *Flavobacterium psychrophilum* strains were cultivated in Anacker & Ordal's medium (0.05% tryptone, 0.05% yeast extract, 0.02% beef extract, and 0.02% sodium acetate; Anacker & Ordal 1955) enriched to 0.5% tryptone; broth and agar (1.2% agar) were both used. The *Flexibacter maritimus* strains were grown in marine 2216E broth (Difco Laboratories, Detroit, MI, USA) and on the corresponding agar (1.2% agar). The *Fx. maritimus* and *Fa. columnare* strains were incubated at 22°C, while the *Fa. psychrophilum* strains were incubated at 18°C. The cultures were harvested during the early stationary phase, approximately 72 h after inoculation (Gherna 1981).

The 7 suspending media tested, each containing a different cryoprotective agent [skim milk (Reichenbach 1989); polyvinylpyrrolidone, dextran, sucrose and horse serum (Gherna 1981); sodium glutamate (Champagne et al. 1991)] are listed below:

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Table 1 Bacterial strains included in this study. JIP: culture collection of the Equipe de Pathologie Infectieuse et Immunité des Poissons, Unité de Virologie et Immunologie Moléculaires, Institut National de la Recherche Agronomique, Jouy-en-Josas, France; LVDL: strain supplied by P. Nougayrède, Laboratoire Vétérinaire Départemental des Landes, Mont de Marsan, France; NCIMB: National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland; LPAA: strain supplied by M. Vigneulle, Laboratoire de Pathologie des Animaux Aquatiques, Centre National d'Etudes Vétérinaires et Alimentaires, Brest, France; FPC: strains supplied by H. Wakabayashi, Dept of Fisheries, Faculty of Agriculture, University of Tokyo, Japan

Species	Strain	Source
Flavobacterium columnare	JIP P11/91	Rainbow trout *Oncorhynchus mykiss*, mouth rot, Ile-de-France, France, 1991
	JIP P06/90	Black bullhead *Ictalurus melas*, skin lesion, Ile-de-France, France, 1990
	LDVL 3414/89	European eel *Anguilla anguilla*, bacterial gill disease, Aquitaine, France, 1989
Flavobacterium psychrophilum	NCIMB 1947^T	Coho salmon *Oncorhynchus kisutch*, kidney, Washington, USA
	LPAA 12023	Rainbow trout, spleen, Brittany, France, 1995
	FPC 839	Ayu *Plecoglossus altivelis*, Tokushima, Japan, 1987
Flexibacter maritimus	FPC 371	Red sea bream *Pagrus major*, Hiroshima, Japan, 1977
	JIP 21/91 (2)	Sea bass *Dicentrarchus labrax*, mouth lesion, Corsica, France, 1991
	JIP 21/91 (3)	Sea bass, skin lesion, Corsica, France, 1991

S1: skim milk.

S2: skim milk, 20%.

S3: Bacto peptone (Difco), 11%; dextran (from *Leuconostoc mesenteroides*, average molecular weight 11300, Sigma Chemical Co., St. Louis, MO, USA), 4%.

S4: lactose monohydrate (Merck, Darmstadt, Germany), 10%; polyvinylpyrrolidone (Prolabo, Paris, France), 0.3%.

S5: dextran (Sigma), 5%; sucrose (= D-saccharose, Prolabo), 5%; sodium L-glutamate (Merck), 1%.

S6: Bacto Brucella Broth (Difco), 67%; horse serum (Gibco BRL, Gaithersburg, MD, USA; inactivated 1 h at 56°C and filtered using a Minisart disposable filter 0.2 µm, Sartorius AG, Göttingen, Germany), 33%.

S7: Bacto Brucella Broth (Difco), 67%; foetal calf serum (Gibco BRL, filtered as above), 33%.

The following procedures were used for preparing the bacterial suspensions:

(1) The broth culture was mixed with an equal volume of each suspending medium. This procedure resulted in a 50% dilution of the cryoprotective agent.

(2) The broth culture was centrifuged (7000 rpm, 4°C, 15 min), the cell pellet was resuspended in 1 ml saline, and 100 µl of this suspension was mixed with 1 ml of each suspending medium.

(3) The colonies were harvested from agar plates with a sterile loop and directly suspended in each medium.

(4) The colonies were harvested from agar plates with a sterile loop and suspended in 1 ml saline, and 100 µl of this suspension was mixed with 1 ml of each suspending medium.

In the case of *Flavobacterium columnare* and *Flexibacter maritimus*, the colonies are very mucoid and adherent to the agar, thus hampering the harvest and suspension of the bacteria. For this reason, methods 1 and 2 were preferred for the *Fa. columnare* strains. The *Fx. maritimus* suspensions were exclusively prepared using method 2, because it freed the cells of the marine broth which, because of its high salt content, might have damaged cell membranes (Champagne et al. 1991) and might have induced thawing of the bacterial suspension before desiccation (Bernardet unpubl. data).

The optical density of the suspensions was spectrophotometrically determined at 525 nm, with the exception of the suspensions prepared with the media S1 and S2 because of their opacity. Agar plates were then inoculated with 50 µl drops of several dilutions of each initial suspension. Colony enumeration showed the bacterial suspensions obtained by method 1 to contain approximately 10^6 cells ml^{-1}, and those obtained by methods 2 to 4 to contain about 10^8 cells ml^{-1}.

For freeze-drying, 300 µl volumes were distributed in glass ampoules (180 × 8 mm) and rapidly frozen at about −65°C in a mixture of dry ice and ethanol on a centrifugal freezer, in order to spread the suspension 0.5 to 1 mm thick. The ampoules were then desiccated for 2 to 3 h on a freeze-dryer (USIFROID, model MSP16, Boulogne, France).

The total number of lyophilization assays performed was 168 with the 3 *Flavobacterium psychrophilum* strains (84 with method 1, 42 with method 3 and 42 with method 4), 84 with the 3 *Flavobacterium columnare* strains (42 with method 1 and 42 with method 2), and 42 with the 3 *Flexibacter maritimus* strains with method 2 only.

In order to assess the efficiency of the process, the bacterial survival rate was evaluated for the different suspending methods and the different suspending media. The ampoules were opened a week after lyophilization and rehydrated by adding 300 µl saline.

Table 2. Average number of viable bacteria per ampoule after freeze-drying in each suspending medium ($\times 10^3$ bacteria per ampoule)

Strain	Medium:	S1	S2	S3	S4	S5	S6	S7
Flavobacterium columnare								
JIP P11/91		0.0	13.2	0.0	0.0	17.2	57.3	57.0
JIP P06/90		0.0	0.4	0.0	0.0	0.5	46.7	30.8
LVDL 3414/89		27.6	1.0	0.0	0.0	8.4	60.0	51.6
Flavobacterium psychrophilum								
NCIMB 1947^T		9.1	4.3	0.0	0.0	0.6	31.8	23.9
LPAA 12023		17.5	17.1	0.0	0.0	12.2	58.4	48.5
FPC 839		25.3	21.8	0.0	0.0	0.9	48.6	58.9
Flexibacter maritimus								
FPC 371		1.6	0.8	0.0	0.0	9.5	60.0	60.0
JIP 21/91 (2)		4.7	30.0	0.0	0.0	0.5	60.0	0.0
JIP 21/91 (3)		4.4	60.0	0.2	0.2	30.0	6.0	1.1

Agar plates were inoculated with 50 µl drops of the pure suspension and with 2 dilutions of the suspension in saline (1/10 and 1/100), and incubated for 72 h at the optimal temperature before colony enumeration.

For the 9 strains and the 7 suspending media the relative efficiency of freeze-drying was calculated according to the following formula:

$$RE = V/V_{max} \times 100$$

where RE is the relative efficiency of a given suspending medium for freeze-drying of a given strain; V is viability rate (= % survival) of this strain freeze-dried in this suspending medium; and V_{max} is the maximal viability rate obtained for this strain among all suspending media tested.

Results and discussion. ***Comparison of the different suspending methods:*** The suspending methods using cells harvested from agar plates (i.e. methods 3 and 4) were tested exclusively with the 3 *Flavobacterium psychrophilum* strains, and no significant difference in cell survival was apparent. On the other hand, the ratio between viability rate of the *Fa. psychrophilum* strains suspended by method 1 and by methods 3 and 4 was 7.43, and the ratio between the viability rate of the *Flavobacterium columnare* strains suspended by methods 1 and 2 was 27.9. In both cases, the first process (method 1) thus seemed to be the most effective. The superiority of method 1 was not definitely demonstrated, because the dilution induced by the suspension method was not taken into consideration. But its convenience from a practical point of view cannot be overlooked. Nevertheless, in the case of *Flexibacter maritimus*, method 2 was preferred for reasons given in 'Materials and methods'.

Comparison of the different suspending media: Table 2 shows the average number of viable bacteria per ampoule after each lyophilisation assay. Fig. 1 shows the relative efficiency of each suspending

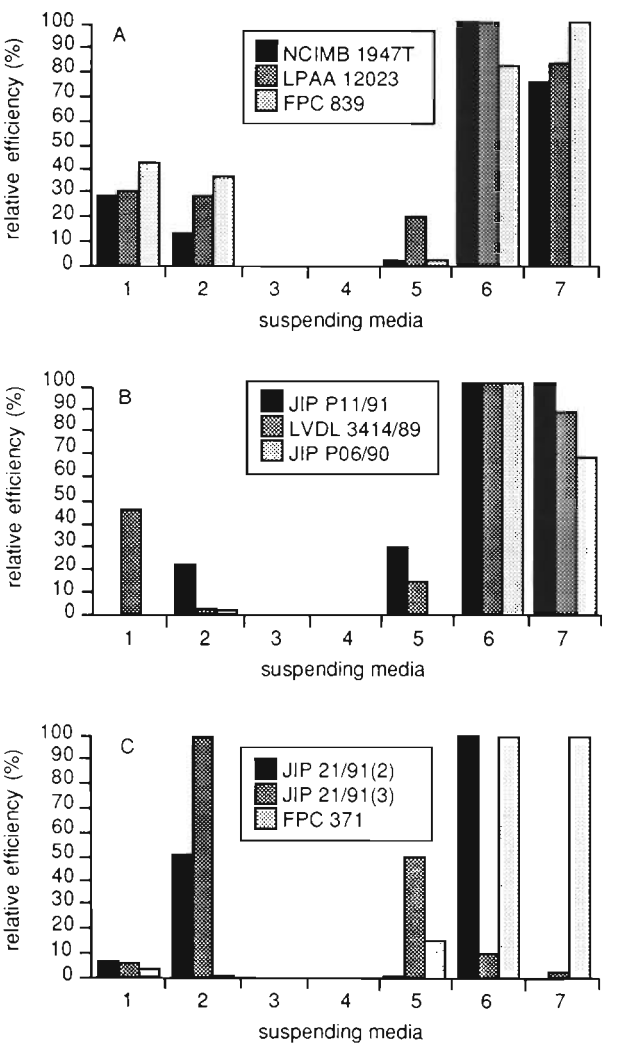

Fig. 1. Relative efficiency of the 7 suspending media tested with 3 strains each of (A) *Flavobacterium psychrophilum*, (B) *Flavobacterium columnare*, and (C) *Flexibacter maritimus*

medium for preserving the 9 strains. The relative efficiency is a better value than the viability rate alone for comparing the efficiency of the suspending media because most viability rates are extremely low and very different from each other. The highest viability rate (19.5%) occurred when the *Flavobacterium columnare* strain LDVL 3414/89 was freeze-dried in suspending medium S6. The media S3 and S4 proved unable to maintain any significant viability of the bacterial strains tested. The media S1, S2, and S5 gave very variable results.The suspending medium S6 was the most efficient except with *Flexibacter maritimus* strain JIP 21/91(3), which was better preserved in medium S2, and with *Flavobacterium psychrophilum* strain FPC 839, which preservation was slightly better in medium S7. Foetal calf serum (medium S7) was compared to horse serum (medium S6), because it significantly increases the growth of *Fla. psychrophilum* when added (5 to 10%) to Anacker and Ordal's medium. This study demonstrated that foetal calf serum is not a better cryoprotective agent than horse serum. A suspension medium including skim milk, sucrose, glucose and bovine serum albumin as cryoprotective agents has recently been proposed for lyophilization of another fish-pathogenic gliding bacterium, *Flavobacterium branchiophilum* (Ostland et al. 1994).

Viability of freeze-dried bacteria depends on numerous factors: suspending method, suspending medium, freeze-drying process, rehydration conditions, and growth and enumeration medium. The suspending medium itself can affect viability of the cells even before freeze-drying. For instance, the viability rate of the 3 *Flavobacterium psychrophilum* strains after suspension in media S3 and S4 was 1/100 of their viability rate in the other media (data not shown). This fact alone probably explains the very low survival obtained with these 2 media. The cultures of *Flexibacter maritimus* are rather labile and degenerative forms (spheroplasts) appear rapidly. It is thus possible that the different results observed among the 3 *Fx. maritimus* strains with certain suspending media were due to slightly different harvesting times.

In conclusion, the results of this study show that the highest viability rate occurred for the 3 bacterial species tested when suspending media containing Bacto Brucella Broth and horse or calf serum were used. This quantitative study should be carried on in order to check if any changes in phenotypical, antigenic or virulence characteristics occurred as a result of the freeze-drying procedures.

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