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Modification of KDM-2 with Culture-spent Medium for Isolation of *Renibacterium salmoninarum*

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ABSTRACT—KDM-2 is a medium widely used for isolation of *Renibacterium salmoninarum* (*R.s.*), the causative agent of bacterial kidney disease (BKD). KDM-2 still has a problem for colonization of *R.s.* at low concentration levels. In the present study, we modified KDM by supplementation of the culture-spent medium of *R.s.* (SMRs) in substitution of FBS. No difference was observed in the growth rate of *R.s.* at ≥ 10^3 cells/mL in KDM broth with 1% FBS regardless of SMRs supplementation. Growth rate of *R.s.* decreased at 10^1 cells/mL of inoculation into KDM with 1% FBS, but it was recovered by supplementation of ≥ 1% SMRs into the medium. The activity of SMRs supporting bacterial growth was stable to treatment at 60°C for 30 min and freezing at −20°C for 7 days. At inoculation of ≤ 300 CFU of *R.s.*, expected colony counts were obtained on the agar plates containing SMRs, while non or less than half of bacteria colonized on the agar plates without SMRs. It was thus considered that the modified KDM by supplementation of SMRs instead of FBS was convenient and inexpensive for isolation of *R.s.*, especially at low concentration levels.

Key words: *Renibacterium salmoninarum*, bacterial kidney disease, BKD, growth rate, colonization

Renibacteium salmoninarum (*R.s.*) is the causative agent of bacterial kidney disease (BKD), a systemic and chronic disease causing high mortality in fingerling and smolt stages of salmonid fish. Outbreaks of BKD were firstly recorded at Atlantic salmon *Salmo salar* in Dee River, Scotland, and chinook salmon *Oncorhynchus tchawytscha* in Columbia River, USA in 1930's (Smith, 1964), and it has been reported in the most areas of the world where salmonid species are cultured (Fryer and Sanders, 1981; Sanders and Barros, 1986). In Japan, outbreaks of BKD have been prevalent among salmonids in wide areas of Japan since first occurrence in 1973 (Kimura and Awakura, 1977; Yoshimizu 1996).

BKD is quite latent and is seldom diagnosed in fish younger than 6−12 month old. BKD-affected fish with are lethargic and dark in color, pop eye, and the belly can be extended due to bloody ascites. Internally there is often milky-reddish ascites, bleeding in the peritoneum and internal organs, and symptom is a slight enlargement of kidney with grey-white nodules spread through the renal tissue filled with soft white liquid. Similar nodules are sometimes observed on the liver, spleen and heart (Smith 1964; Wiens and Kaattari, 1999).

R.s. is a gram-positive diplococcobacillus with 0.3~1.0 × 1.0~1.5 μm in sizes, and grows very slowly due to fastidious nutritional requirements (Ordal and Earp,1956; Austin *et al*., 1983; Daly and Stevenson, 1993; Evelyn 1977; Starliper *et al*., 1998; Teska 1994; Benediktsdóttir *et al*., 1991). *R.s.* produces abundant quantities of an extracellular protein with 57 kDa, which is called p57 or major soluble antigen (MSA) and associated with the bacterial surface (Bruno 1988; Getchell *et al*., 1985; Turaga *et al*., 1987). It is considered that MSA is a significant virulence determinant, because *R.s.* isolates with reducing expression of MSA genes showed lower mortalities upon infection (Bruno, 1988; Senson and Stevenson 1999; O'Farrell *et al*., 2000; Coady *et al*., 2006), whereas some isolates of *R.s.* possessing an additional MSA gene showed more virulent (Rhodes *et al*., 2004).

A kidney disease medium containing 0.1% cysteine and 20% fetal bovine serum (FBS) has been devised as KDM-2 by Evelyn (1977) for culture of *R.s*. The serum component can be replaced by charcoal (Daly and Stevenson, 1985). It was also reported that growth of *R.s.* is enhanced by a heavy inoculum of a "nurse culture" in the centre of KDM-2 agar plates or addition of 5% spent media to the agar medium (Evelyn *et al*., 1989, 1990; Teska 1994). From those observation, it is considered that the enhancement of *R.s.* growth is presumably due to the action of a diffusible factor, which is able either to inactivate a toxic component in the medium or to stimulate growth of the bacterium although those mechanisms are still not clear (Wiens and Kaattari, 1999).

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In the present study, we focused on the "nurse culture" reported by Evelyn *et al*. (1989, 1990) and the bacterial spent medium (Teska, 1994) to investigate how the culture-spent medium of *R.s.* (SMRs) influence to bacterial growth in the broth medium. Furthermore, the required quantity and stability of the SMRs for enhancement of bacterial growth were investigated. Finally, it was demonstrated that *R.s.* at a low concentration level was suitable to colonize in the modified KDM agar containing SMRs rather than KDM-2 agar derived by Evelyn (1977).

Materials and Methods

Bacterial strain and a culture-spent medium

R.s. Tok46 isolated from kidney of BKD-affected masu salmon *O. masou* reared in Tokushibetsu hatchery, Hokkaido in 2001 was used in the present study. *R.s.* was inoculated at OD_{620}: 0.005, approximately 10^7 cells/mL, into a modified KDM broth medium (1% peptone, 0.05% yeast extract, 0.1% cysteine, pH 6.6) containing 1% fetal bovine serum (FBS) [KDM-FBS (1%)], and incubate with shaking (100 rpm) at 15°C for 5–6 days until OD_{620}: 0.5, where *R.s.* was in a logarithmic growth phase. The viable cell count of *R.s.* at a logarithmic growth phase with OD_{620}: 0.5 was approximately 10^9 cells/mL (Okuda *et al*., 2008). The culture-grown bacteria was washed three times with sterilized saline solution by centrifugation (12,000 × *g*, 5 min, 4°C), and resuspended with sterilized saline solution at adequate bacterial concentration for the following experiments.

R.s. was inoculated to KDM-FBS (1%), and incubated at 15°C until OD_{620}: 0.5. The culture broth was centrifuged (12,000 × *g*, 5 min, 4°C) and then filtered through a membrane with a pore size of 0.45 μm (Advantec) to obtain the culture-spent medium of *R.s.* (SMRs).

It was confirmed in our preliminary study that *R.s.* grew in the KDM-FBS (1%) as well as in the KDM-2 (Evelyn, 1977), but grew very slow in the KDM-FBS (0%) (data not shown). Thus, we decided to use the KDM-FBS (1%) broth as a standard medium in the following experiments.

Influence of SMRs to growth rate of R.s.

The SMRs was added at 1% or 0% (control) to KDM-FBS (0%) and -FBS (1%) broth. *R.s.* was inoculated at 10^1, 10^3 or 10^5 cells/mL into those media, and incubated at 15°C for 30 to 70 days with shaking (100 rpm). At adequate days after the bacterial inoculation, a portion of each cultured medium was collected to monitor the bacterial turbidity (OD_{620}).

Stability of the growth factor in SMRs

The SMRs was treated by treatment at 60°C for 30 min, freezing at −20°C for 7 days, or incubation at 4°C for 7 days (control), and the each treated SMRs was added at 1% into the KDM-FBS (1%) broth. *R.s.* at 10^1 cells/mL was inoculated into each KDM-FBS (1%) broth containing the treated SMRs and also into KDM-FBS (1%) broth without SMRs supplementation, and incubated at 15°C for 50 days with shaking (100 rpm). At adequate days after the bacterial inoculation, a portion of each cultured medium was collected to monitor the bacterial turbidity (OD_{620}).

Colonization of R.s. *in KDM-2 agar plate containing SMRs*

Four condition of KDM agar plates containing different concentration of FBS and/or SMRs were prepared as follows; 1) containing 10% FBS and 2% SMRs, 2) containing 10% FBS but without SMRs supplementation, 3) containing 2% SMRs but without FBS supplementation, 4) containing neither FBS nor SMRs. *R.s.* was spread at 12, 60 and 300 CFU/plate on those agar plates with two replicates, and incubated at 15°C for 28 days to count colonies of *R.s.* on the plates.

Growth curves of R.s. *at a low concentration level in the broth media with SMRs*

R.s. was inoculated at approximately 50 CFU/mL into KDM-FBS (1%) broth containing 0% and 1% SMRs, and incubated at 15°C for 30 to 70 days with shaking (100 rpm). At adequate days after the bacterial inoculation, a portion of each cultured medium was collected and spread onto the KDM agar plate containing 2% SMRs. The agar plates were incubated at 15°C for 50 days to monitor the viable cell count of *R.s*.

Results and Discussion

Influence of SMRs to growth of R.s.

Change of *R.s.* turbidity in the KDM-FBS (1%) broth containing 0 and 1% SMRs was shown in Fig. 1. When *R.s.* were inoculated at 10^5 cells/mL into the KDM-FBS (1%) containing 0% or 1% SMRs, bacterial turbidities began to increase from 11 days, and reached OD_{620}: > 1.0 within 14 days regardless of SMRs supplementation (Fig. 1A). By inoculation at 10^3 cells/mL, turbidities of those media began to increase from 20−21 days, and reached OD_{620}: > 1.0 within 23 days, likewise regardless of SMRs supplementation (Fig. 1B). In the inoculation at 10^1 cells/mL, turbidity of the KDM-FBS (1%) containing 1% SMRs began to increase from 26 days and reached to OD_{620}: > 1.0 at 29 days, while that of the medium without SMRs supplementation began to increase at 35 days, and reached OD_{620}: > 1.0 at 38 days (Fig. 1C). These results demonstrated that no significant difference in growth rate of *R.s.* at > 10^3 cells/mL of inoculation regardless of SMRs supplementation, however, at the inoculation with 10^1 cells/mL, the bacterial growth in the media without SMRs supplemen-

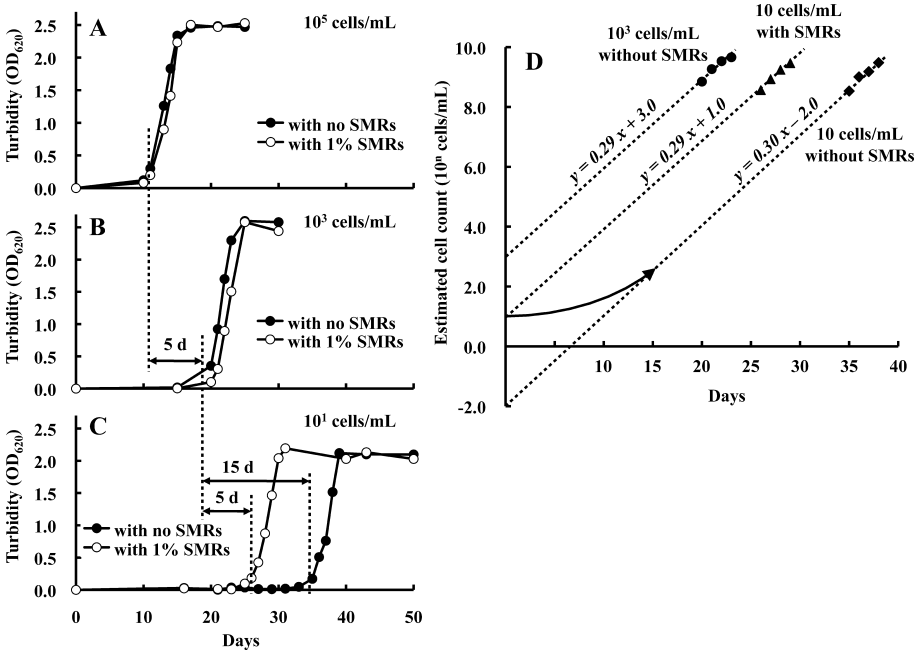


Fig. 1. Growth curves of *R.s.* in KDM-FBS (1%) broth containing 0% and 1% culture-spent medium of *R.s.* (SMRs). A: *R.s.* was inoculated at 10^5 cells/mL of initial dose, B: at 10^3 cells/mL, C: at 10^1 cells/mL, D: semi-logarithmic graph for the regression lines of *R.s.* growth in KDM-FBS (1%) broth containing 0% or 1% SMRs.

tation was clearly slower than that in the media supplemented with SMRs.

The time when the media began to become turbid was delayed by decreasing of inoculated *R.s.* doses, however, *R.s.* required 5–6 days to grow 10 time from OD_{620}: 0.2 to 2.0 regardless of inoculation dose and of SMRs supplementation (Fig. 1A–C). Thus, the represented regression lines for *R.s.* growth in the broth media at 10^1 or 10^3 cells/mL of inoculation were shown in a semilogarithm graph (Fig. 1D). A regression line for the bacterial growth at 10^3 cells/mL of inoculation in the medium without SMRs supplementation was "y = 0.29 x + 3.0", while that with 10^1 cells/mL in the medium supplemented with SMRs was "y = 0.29 x + 1.0", indicating that the intersection points of those regression lines with the vertical axis accorded with the initial dose of *R.s.* inoculated to each medium. However, the regression line for the bacterial growth at 10^1 cells/mL of initial inoculation in the medium without SMRs supplementation was "y = 0.30 x – 2.0", and its intersection point with the vertical axis were 10^{-2} cells/mL. It was thus considered that the *R.s.* inoculated at 10^1 cells/mL into the media with SMRs supplementation was able to propagate with cell division at constant rate, while the *R.s.* inoculated into the media without SMRs supplementation at 10^1 cells/mL might propagate slowly until 10^3 cells/mL as shown as the arrow line in Fig. 1D, because *R.s.* inoculated at 10^3 cells/mL into the medium without SMRs supplementation was able to propagate at constant rate (Fig. 1D). Incidentally, those three regression lines showed the same inclination coefficient as 0.3, meaning that the bacterial generation time was cal-

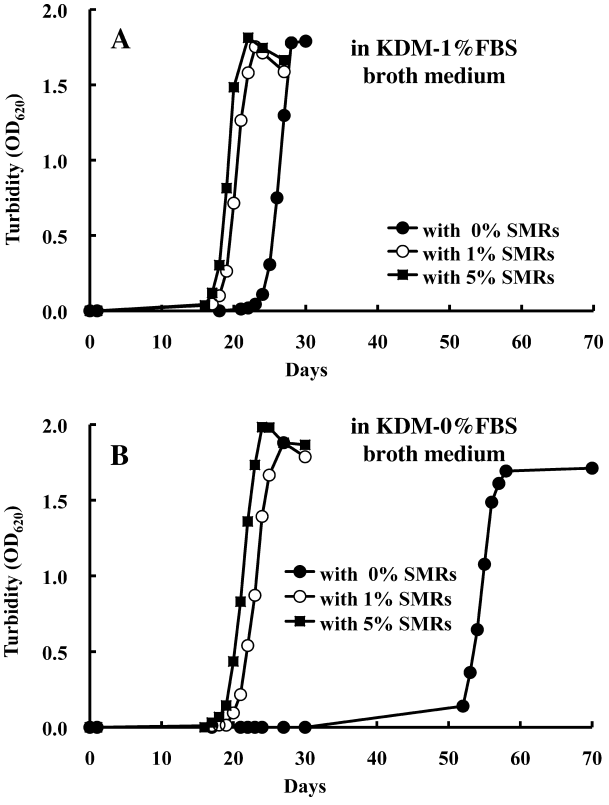


Fig. 2. Influence of concentration of FBS and SMRs to *R.s.* growth. A: in KDM-FBS (1%) broth containing different concentration of SMRs, B: in KDM-FBS (0%) broth containing different concentration of SMRs.

culated as approximately 24 h, which was consistent with the previous data (Fryer and Sanders 1981; Wiens and Kaattari, 1999; Okuda *et al*., 2008). From these results, we speculated that *R.s.* grew at constant rate of cell division at a high concentration level, but its cell division rate decreases drastically in a low concentration level at < 10^3 cells/mL. Moreover, the decreased division rate could be recovered by SMRs supplementation.

Influence of SMRs concentration to R.s. *growth*

When *R.s.* was inoculated at 10^1 cells/mL, the bacteria in the KDM-FBS (1%) containing 0%, 1% and 5% SMRs grew up to OD_{620}: > 1.0 within 26, 20 and 19 days, respectively (Fig. 2A). At 10^1 cells/mL of inoculation, *R.s.* in the KDM-FBS (0%) containing 0%, 1% and 5% SMRs required 55, 24 and 21 days to grow up until OD_{620}: > 1.0, respectively (Fig. 2B). These results demonstrated that *R.s.* required additional 6–31 days to grow under the SMRs absence rather than the SMRs presence in the medium, suggesting that SMRs influences to growth of *R.s.*, especially in a medium containing no FBS, moreover 1–2% of SMRs supplementation is good enough to support growth rate of *R.s.* at a low concentration level.

Stability of SMRs activity

As shown in Fig. 3, *R.s.* required 22–24 days to grow up to OD_{620}: > 1.0 in the KDM-FBS (1%) broth containing SMRs treated at 60°C for 30 min, at −20°C for 7 days or at 4°C for 7 days (control), meaning there was no difference among them. However, *R.s.* in the medium without SMRs supplementation required 36 days to grow up to the same level, and which was clearly slower than those in the media containing the treated SMRs. From these results, it was revealed that SMRs activity supporting growth of *R.s.* was stable against heat and freezing treatment, suggesting that SMRs seems to be convenient to supplement into culture media and to store long-term.

Influence of SMRs for colonization of R.s.

R.s. is generally known to grow slowly due to fastidious nutritional requirements, and which causes in the difficulty of colonization on KDM-2 agar, especially when a low concentration level of *R.s.* (approximately less than 10^3 CFU/plate) were spread. The present results demonstrated that some of the fastidious nutrition required for *R.s.* could be contained in SMRs. Thus, we presumed that the modified KDM agar plates by SMRs supplementation could become useful and convenient for colonization of *R.s.* at a low concentration level. To verify the presumption, 300, 60 and 12 CFU of *R.s.* were spread on KDM agar supplemented with and without FBS and/or SMRs to compare *R.s.* colonization (Table 1). As the results, *R.s.* was colonized 263–289 CFU/plate, 49–53 CFU/plate and 10–23 CFU/plate in the KDM agar plates supplemented with 2% SMRs regardless of FBS supplementation, respectively. However, only 129 CFU out of the spread 300 CFU of *R.s.* were colonized on the KDM-FBS (10%) without SMRs supplementation, moreover, no colonization was observed on the agar plate spreading at 60 or 12 CFU/plate of *R.s*. Also, no colonization was observed on the KDM-FBS (0%) plates without SMRs supplementation, even though 300 CFU of *R.s.* were spread (Table 1). These results suggest that colonization at a low concentration level of *R.s.* will be possible by using the KDM-SMRs agar plate, for examples, for viable cell count of *R.s.* and for culture isolation from BKD-affected fish under a latent state. Moreover, the modified KDM supplemented with SMRs in substitution for FBS is quite inexpensive rather than KDM-2 devised by Evelyn (1977).

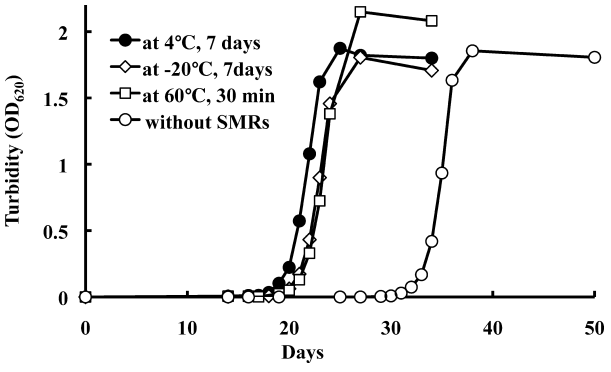


Fig. 3. Stability of SMRs activity.

Table 1. Influence of SMRs for colonization of *R.s.* on the KDM agar plates

Medium		Colonies on agar plates*1 (CFU/mL)		
Culture Supernatant	FBS			
2%	10%	263	49	10
2%	0%	289	53	23
0%	10%	129	0	0
0%	0%	0	0	0
Spread cells*2		300	60	12

*1: Average of *R.s.* colonized on two agar plates.
*2: Estimated cell count for spread bacteria on the agar plates.

Verification of growth rate of R.s. *at a low concentration level*

Finally, growth pattern of *R.s.* at a low dose in broth media was confirmed by colonization using the modified KDM agar plate with SMRs supplementation (Fig. 4). The growth of *R.s.* in the broth medium supplemented with 1% SMRs was shown by a regression line "y = 0.46 x + 1.7", while that in the broth medium without SMRs supplementation was by the separated two

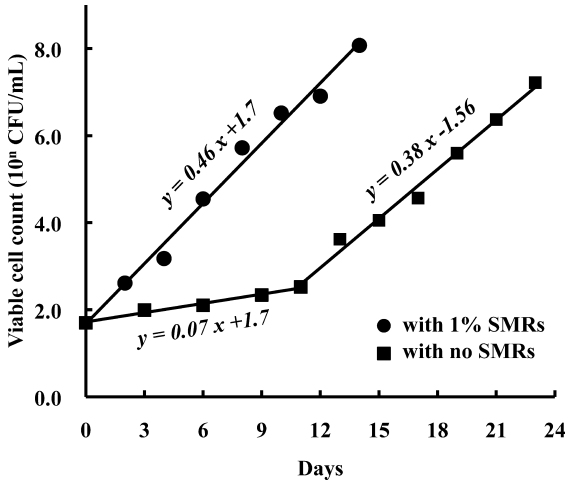


Fig. 4. Growth curves of *R.s.* in KDM-FBS (1%) with supplementation of 0% and 1% SMRs. The viable cell count in each medium were monitored using the modified KDM agar plates supplemented of 2% SMRs.

regression lines, "y = 0.07 x + 1.7" and "y = 0.38 x – 1.56" (Fig. 4). These results demonstrate that growth rate of *R.s.* decrease at a low concentration level (< $10^{2.5}$ cells/mL), and it was recovered by addition of SMRs into culture medium, i.e. our hypothesis shown in Fig. 1D was experimentally confirmed. And also the modified KDM agar plate with SMRs supplementation was evaluated for colonization of *R.s.* at a low concentration level. Okuda *et al*. (2008) reported a detection limit of *R.s.* by PCR and IFAT was approximately 10^5 cells/50 mg tissue, whereas the modified KDM-SMRs became possible for quantitative detection of *R.s.* at more low concentration levels.

Unfortunately, promoting mechanism of the SMRs support *R.s.* growth rate did not become clear in the present study. As described above, the enhancement of *R.s.* growth is presumably due to the action of a diffusible factor, which is able either to inactivate a toxic component in the medium or to stimulate growth of the bacterium although those mechanisms are still not clear (Wiens and Kaattari, 1999). And we believe that *R.s.* require a "cell to cell" communication to each other for their growth, especially at a low concentration, and the SMRs may includes the cell communication substance(s), which is equivalent to the diffusible factor of the "nurse culture" reported by Evelyn *et al*. (1989, 1990). In our preliminary studies, an extracellular protein with 57 kDa (MSA) of *R.s.* was considered to have an important role with the SMRs activity, because only MSA was detected from SMRs as a protein derived from *R.s.* by western blot analysis with antiserum against *R.s.* (data not shown).

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