Anti−angiogenic, Anti−cell Adhesion Switch from Halophilic Enterobacteria

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Abstract   The halophilic enterobacteria, *Enterobacteria cancerogenus*, was isolated from the intestines of the fusiform fish (*Trachurus japonicus*) to yield a protein-like material termed PLM-f74. PLM-f74 was characterized by strong inhibition ratios to angiogenesis (82.8% at the concentration of 18.5 μg/mL) and elevated antioxidative capacities with low toxicity. The PLM-f74 is a glycoprotein comprised of saccharides and amino acids. PLM-f74 inhibited non-activated U937 monocytic cell adhesion to HUVECs activated with IL-1β by 78.0%, and the adherence of U937 cells treated with the PLM-f74 and stimulated with IL-1β to unstimulated HUVECs decreased by 102%. When both cell types were pretreated with PLM-f74, the adhesion of U937 cells to IL-1β stimulated HUVECs was completely suppressed by 121% at a concentration of 18.5 ug/mL. PLM-f74 blocked signal pathways from VEGFR2, PI3K, β-catenin and VE-cadherin to NF-kB based on western bolt analysis. And also inhibited IL-1-stimulated HUVEC expression of the adhesion molecules, ICAM-1 by 40%, VCAM-1 by 60%, and E-selectin by 70% at the same concentration noted above. New anti-angiogenic and anti-cell adhesion materials showing elevated antioxidative capacities and non-toxicity may be expected from these results.

Key words : Anti-angiogenesis, antioxidative capacity, cytotoxicity, fusiform fish, cell-adhesion, protein-like material (PLM)

Introduction

Angiogenesis is the process of forming new vasculogenesis from blood vessels [28,37] and occurs in the healthy body to heal wounds and restore blood flow to tissues after injury [2,27]. In females, angiogenesis also occurs during monthly reproductive cycles to rebuild the uterine lining [25,32,34], to mature the ovum during folliculogenesis and ovulation [1,3] andduring pregnancy to develop the placenta and aid circulation between the mother and developing fetus [15-16,38]. When angiogenic growth factors are produced in excess of angiogenesis inhibitory factors, the balance is tipped in favor of vasculature growth, and when inhibitors are present in excess of stimulators, angiogenesis is arrested. Normal, a healthy body maintains a perfect balance of the angiogenesis modulators. In general, angiogenesis is turned off by the production of more inhibitors than stimulators [36]. The body loses control over angiogenesis in many serious states of disease. Angiogenesis-dependent diseases result when new blood vessels either grow excessively or insufficiently. Excessive angiogenesis is associated with diseases such as cancer [23], diabetic blindness [17], age-related macular degeneration [12], rheumatoid arthritis [19], and psoriasis [30] because the new blood vessels feed the diseased tissues at the expense of the normal tissues which are destroyed in the process [33]. In the case of cancer, the new vessels allow tumor cells to escape into the circulation and seed other organs i.e. metastasize [13]. Alternatively, as a result of insufficient angiogenesis, coronary artery disease[11], stroke [20], and

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delayed wound healing [4] will occur because of the inadequate growth of blood vessels and a failure to re-store proper circulation which leads to the increased risk of tissue death [29].

In cases of the excessive angiogenesis, there is a de-pendence upon "on" switches, known as angio-genesis-stimulating growth factors [9], e.g. angiogenin, angiopoietin-1, interleukin-8 (IL-8), tumor necrosis fac-tor-alpha (TNF-alpha), and vascular endothelial growth factor (VEGF), and for the insufficient case, it depends on "of" switches, known as angiogenesis inhibitors [6], e.g. angiostatin, the interferon's (alpha, beta, and gam-ma), interleukin-12, retinoid, and transforming growth factor-beta (TGF-β).

Many additional switches likely will be found in na-ture including among animals, plants [24] and prokaryotes. Currently, novel and inexpensive anti-an-giogenic switches have been under investigation for curing cancer, and ideally without side [18]. A major problem with such anti-angiogenic switches at present is that it is very difficult to obtain a sufficient amount of material from animal and plant resources.

While marine organisms, including marine bacteria, have been very useful in providing materials for human health, they vary greatly from their terrestrial counterparts. These differences are likely because ma-rine organisms have adapted in a more extreme environ-ment than that of terrestrial bacteria in terms of nu-trients, salt levels, a higher osmotic pressure, etc. From these reason, new components bearing anti-angiogenic effects will be expected from these marine organisms. Eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA) from fusiform fish like Trachurus japonicus are known as inhibitors of cancer cell growth and of athero-sclerosis [5,26]. These unsaturated fatty acids are not oxidized in the body of fusiform fishes due to unknown factors which prohibit fatty acid oxidation. From these reason, it's a valuable work searching new switches from halophilic bacteria which is a different environ-ment compared to a land bacteria with novel anti-angio-genic effects and with reduced toxicity. Because bac-teria were more simple to get a large amount of such substances through fermentation.

E. cancerogenus, as the culture time increased, the ORP values decreased to a reduced category of -0.229 Volts, and thus, were designated as having higher anti-oxidative capacity. ORP values were decreased fastly to the reduced category shown in Fig. 1, it was seemed
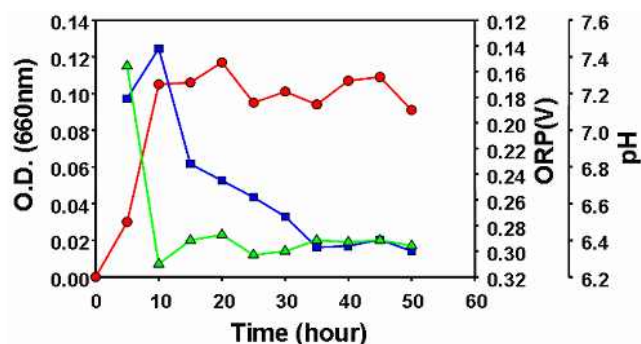

Fig. 1. Time course of biomass, pH, and ORP values of hal-ophilic bacteria.
●: Optical density at 660 nm   ■: ORP values   ▲: pH

to be synthesized some materials which carrying higher antioxidative capacity such as protein like materials.

Protein-like material (PLM) taken from *E. cancer-ogenus*(designated as PLM-28) for their anti-angiogenic effects had excellent anti-angiogenic effects (96.8% and 57.6%) as indicated from Fig. 2.

The lyophilized PLM-28 was subjected to SEC using a column with 2cm x 150cm(D x H) packed with Sephadex G-100, with a flowing phosphate buffer (pH 7.0). The protein like material was measured at 280 nm with a spectrophotometer, as shown in Fig. 3.

The antioxidative capacity for the fractions numbered 27, 62, 66, 71, 74, 78, 82 and 86 from PLM-28, was measured by the FRAP method. Fractions numbered 62, 71, 74 and 82 showed the highest antioxidative capaci-ties EGCG 25uM was included as a positive control as shown in Fig. 4.
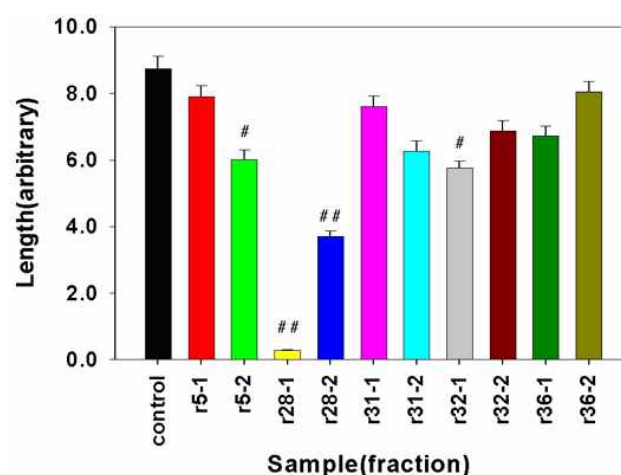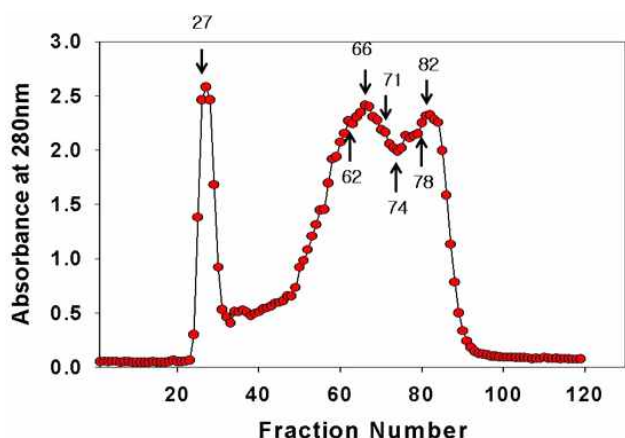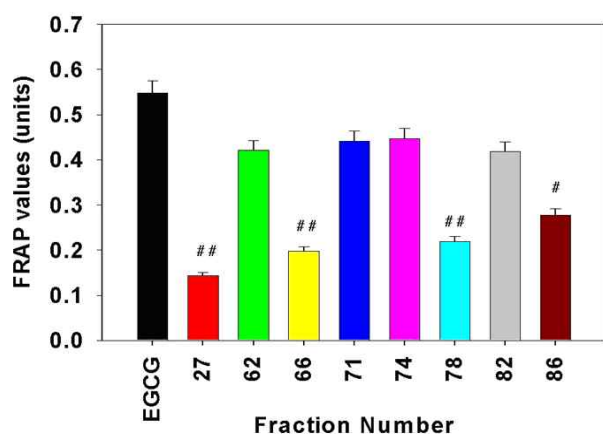

Fig. 2. Screening of strains bearing anti-angiogenesis effect from fusiform fish. Data are the mean±S.D. of three experiments. P## 〈0.01, P# 〈0.05 compared with control.

Fig. 3. A280 vs. fraction number of PLM from *E. cancerogenus* with size exclusion chromatography.

Fig. 4. Antioxidative capacity of fractions from PLM-28, and 25 uM-EGCG as a positive control. Data are the mean±S.D. of three experiments. P## ⟨0.01, P# ⟨0.05.

Fractions numbered 74 and 82 shown in Fig. 3, 4 expressed higher anti-angiogenesis effects due to their antioxidative capacities.

Fig. 5 indicates the results when these effective fractions were tested for anti-angiogenesis effects with different concentrations of PLM. The PLM-28 from fraction number 74 (PLM-f74) showed strong inhibition ratios of angiogenesis as 82.8%, 65.9%, 30.2%, and 22.3%fraction number 82 exhibited 67.3%, 48.0%, 15.6%, and 3.3% at the concentration of 18.5 ug/mL, 7.4 ug/mL, 3.7 ug/mL and 0.74 ug/mL, respectively. Low molecular weight PLM (No. 74) had a higher anti-angiogenesis effect than that of the high molecular weight PLM (No. 27). It was assumed that the low molecular weight PLM would inhibit the interaction of immune and endothelial cells, which is mediated through the endothelial expression of cell surface adhesion mol-
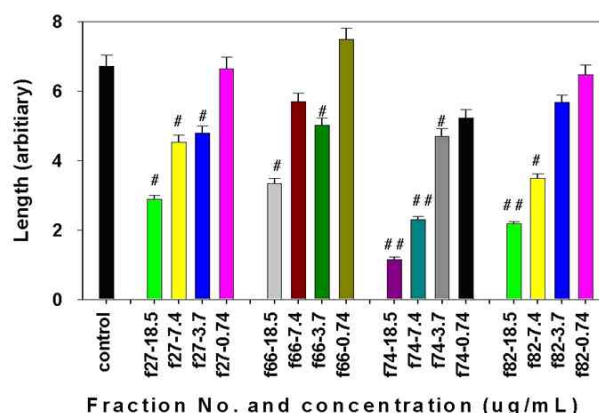

Fig. 5. Anti-angiogenic effect of PLM-28 with effective fraction on their concentration. Data are the mean±S.D. of three experiments. P## ⟨0.01, P# ⟨0.05 compared with control.

ecules and ligands, thereby acting as receptor molecules [13]. While PLM-f82 exhibited lower anti-angiogenic effect than PLM-f74, although PLM-f82 was lower molecular than PLM-f74, it would be seemed that some suitable structure for interaction between PLM and receptor molecules [11].

When toxicity for the four types of effective fractions numbered 27, 66, 74, 82 was examined different concentrations of PLM-28, fraction numbers 74 and 82 expressed lower toxicity as shown in Fig. 6. The toxicity of a compound occurs by the uptake of the compound by the cell or through interaction with the cell membrane and associated molecules, so that a higher molecular weight fraction (27th or 66th fraction) would likely be taken up less than the lower molecular weight fractions (74th or 82nd).
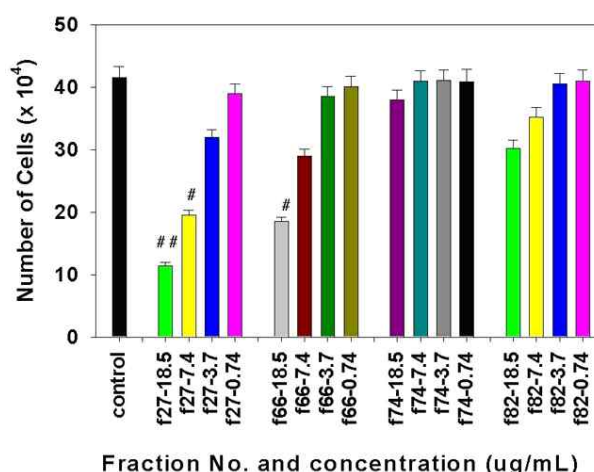
Fig. 6. Cytotoxicity with PLM-28 fraction against HUVECs. Data are the mean±S.D. of three experiments. P## ⟨0.01, P# ⟨0.05 compared with control.
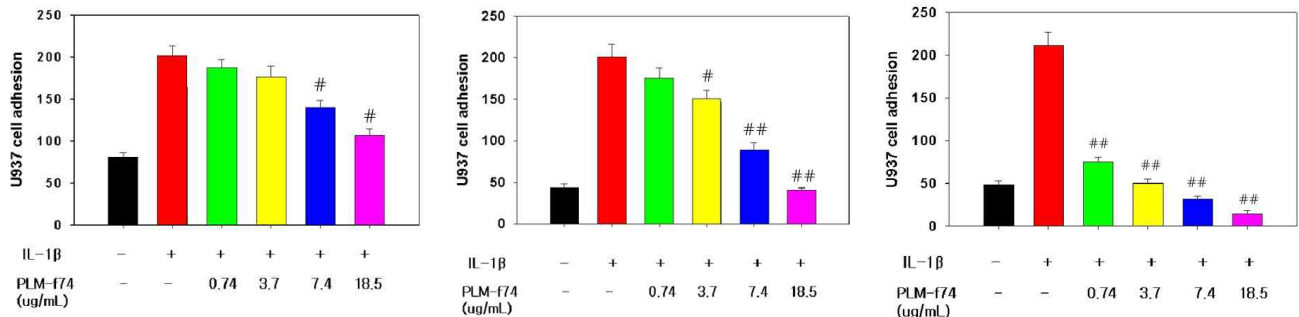
Fig. 7. The effect of PLM-f74 on inhibition of U937 cell adhesion to HUVEC with or without IL-1β (a), U937 stimulated with IL-1β cell adhesion to HUVEC (b), and both cells were incubated with PLM-f74 and HUVECs were stimulated with IL-1β. Data are the mean±S.D. of three experiments.

U937 cell adhesion to IL-1β stimulated HUVEC: The monocyte-like U937 cells did not adhere significantly to unstimulated HUVECs (Fig. 7a). When HUVEC was stimulated with IL-1β, however, adhesion increased significantly. This stimulated adhesion was clearly inhibited by PLM-f74, and in a dose-dependent manner. PLM-f74 at 0.74, 3.7, 7.4, and 18.5 ug/mL suppressed U937 cell adhesion by 12.1, 21.2, 50.9, and 78.2%, respectively.

IL-1β stimulated U937 cell adhesion to HUVECs: U937 cells were treated with the PLM-f74 at different doses and stimulated with IL-1'β for 2h then tested for their adherence to untreated and unstimulated HUVECs (Fig. 7b). A dose-dependent decrease in adhesion (by 15.8, 31.9, 70.8, and 102%) was observed in U937 cells treated with the same concentrations as above, respectively. The inhibitory effect of PLM-f74 on the reduction of U937 cell adhesion to HUVECs was more pronounced when U937 cells were treated with PLM-f74 compared to when HUVECs were treated with these PLM-f74.

U937 cell adhesion to HUVECs when both cells were treated with PLM-f74: The adhesion of U937 cells to IL-1-stimulated HUVECs was markedly decreased when both cell types were pretreated with PLM-f74 (Fig. 7c). PLM-f74 dose dependently (p<0.01) inhibited the adhesion of U937 cells to HUVECs by 83.7, 99.2, 110, and 120.8% at the same concentrations as above, respectively.

Cell adhesion is very important for cell morphogenesis, cell maintenance, and tumor metastasis. Because PLM-f74 significantly suppressed cell adhesion induced by IL-1β, which regulates cell surface adhesion molecules and the adhesion of circulating monocytes to the arterial endothelial lining, PLM-f74 could be very useful in preventing metastasis and atherosclerosis.

To determine whether the PLM-f74 inhibition of tubular formation in HUVEC is through suppression of the signal pathways of VEGFR-2, PI3K, β-catenin, and VE-cadherin, the expression of signal molecules was assessed by use of anti-signal molecule antibodies such as anti-VEGFR-2 (Flk-1), anti-PI3K, anti-β-catenin, and anti-VE-cadherin, respectively. As shown in Fig. 8, VEGF significantly increased the signal molecules, and pre-supplementation of HUVECs with dose dependent dosages of 0.74 and 18.5 ug/mL, PLM-f74 inhibited expression of all four typesof signal molecules. Finally, because PLM-f74 suppressed the pathways from four kinds of signal molecules to NF-kB, angiogenesis would be decreased. Especially, VE-cadherin is an important molecule in cell-cell recognition and also to strengthen cell contact during vascular morphogenesis [31].
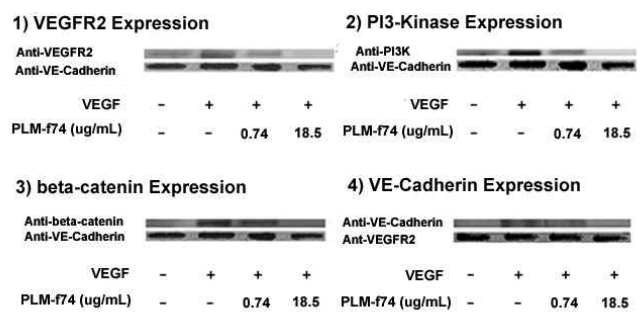
Fig. 8. Effect of PLM-f74 on interaction of VE-cadherin with VEGFR-2, PI3-kinase, and β-catenin upon cell activation with VEGF. HUVEC cell extracts were immuno-precipitated with VE-cadherin antibodies (1-3) and immunoblotted with antibodies to VEGFR-2, PI3-kinase, and β-catenin, IP with VEGFR2 (4) and IB, VE-cadherin. Cells were starved during supplementation for 24h before stimulation with VEGF(50 ng/mL).
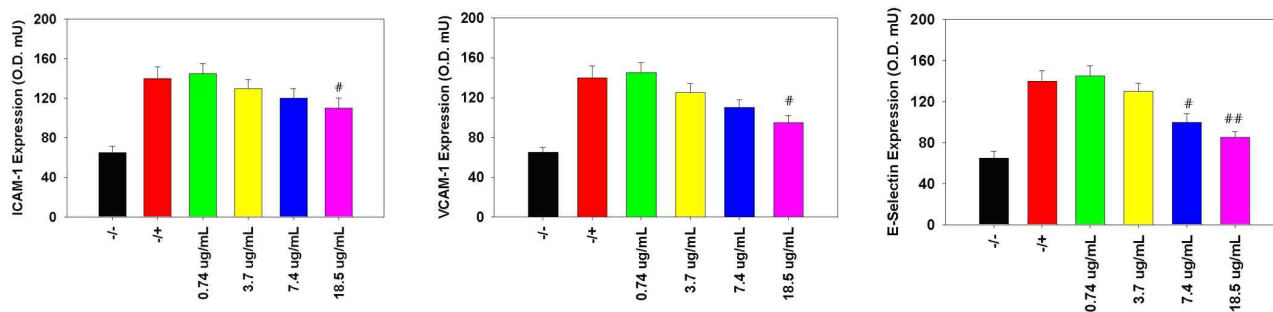
Fig. 9. Inhibition of endothelial cell adhesion molecule expression by PLM-f74 at different doses, ICAM-1(a), VCAM-1(b), and E-selectin(c). are the means. of three experiments. P## ⟨0.01, P# ⟨0.05 compared with control.

Recently, Carmeliet et al. [7] reported that VE-cadherin molecules are also involved in VEGF signaling for vascular endothelial cell survival and proliferationin angiogenesis. VE-cadherin and its associated molecule, β-catenin, chemically cross-link with VEGFR2-PI3kinase and support the survival of endothelial cells and the development of new capillaries. VEGF, which has been shown to be a major factor in tumor growth and other growth factors produced from tumor cells and blood vessel cells, plays an important role in expansion of the microvascular network needed to supply oxygen and nutrients for the rapid growth of tumor masses [14].

PLM-f74 reduced IL-1β stimulated HUVEC expression with dose dependence, and showed a significant inhibition with ICAM-1 by 40%, VCAM-1 by 60%, and E-selectin by 73% at the concentration of 18.5 ug/mL.

Our data demonstrating a reduction of IL-1β monocyte adhesion by PLM-f74 and a decrease of ICAM-1 production by HUVECs can be interpreted to support important roles of use for early events in atherosclerosis. Further, IL-1β induced expression of VCAM-1 by HUVECs has been reported to be reduced by antioxidants such as N-acetyl cystein [21]. α-Tocopherol, has also been reported to inhibit adhesion of the U937 monocyte cell to HUVECs when stimulated with agonists such as IL-1β [13]. PLM-f74 had higher antioxidative and capacity. Also it inhibited IL-1β and induced ICAM-1, VCAM-1, and E-selectin (Fig. 9). Similar results were identified by Faruqui et al [13], who reported that IL-1β induced monocyte adhesion was correlated with reduced expression of E-selectin by HUVECs. Because IL-1β regulates the expression of specific proteins involved in the adhesion and subsequent migration of leukocytes into tissues [9], PLM-f74 could have an important role in the inhibition

of angiogenesis and atherosclerosis based on IL-1β actions.

Because the photograph of this strain showed polysaccharide like material on its cell wall from Fig. 10. While an analysis of ion chromatography, three types of saccharides were detected: D-glucose, D-arabinose, and lactose. Amino acid analysis indicated 18 components: Glu, Amm, Met, Arg, Leu, His, Lle, Ser, Ala, Lys, Gly, Tyr, Thr, Val, Phe, Asp, Cys, and Pro. PLM-f74 is assumed to be glycoprotein, because of the identification of saccharides and amino acids, and no lipids by hexane extraction. Halophilic enterobacterium, E. cancerogenus was showed higher anti-oxidative capacity, and anti-angiogenic effects with PLM-f74 (protein-like material from the 74th fraction of SEC), comprised of saccharides and amino acids, showed strong inhibition ratios of angiogenesis e.g. 82.8%, 65.9%, 30.2%, and 22.3% at the concentrations of 18.5 ug/mL, 7.4 ug/mL, 3.7 ug/mL and 0.74 ug/mL, respectively. PLM-f74 also controlled non-activated U937 monocyte cell adhesion to HUVECs activated with IL-1β by 78.0% at the concentration of 18.5 ug/mL. Adherence
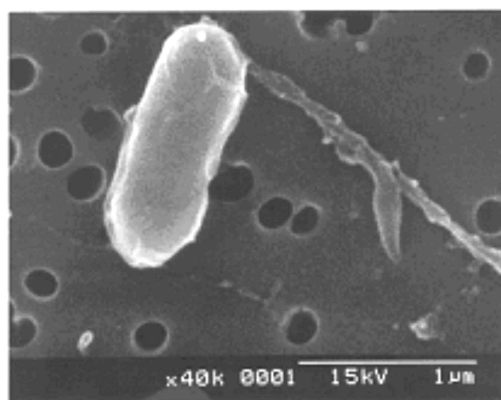
Fig. 10. SEM photograph of E. cancerogenus with unidentified material like polysaccharide.
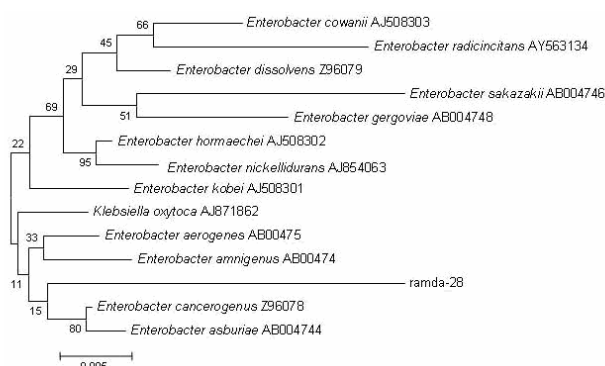
Fig. 11. Identification of *E. cancerogenus* with 16s rDNA sequencing.

of U937 cells treated with PLM-f74 and stimulated with IL-1β to unstimulated HUVECs decreased by 102% at the same concentrations.

When both cell types were pretreated with PLM-f74, the adhesion of U937 cells to IL-1β stimulated HUVECs was completely suppressed i.e. 98.0%, 99.3%, 110%, and 121% at the concentrations of 0.74 ug/mL, 3.7 ug/mL, 7.4 ug/mL and 18.5 ug/mL, respectively. PLM-f74 suppresses angiogenesis because the signal pathways from signal molecules of VEGFR-2, PI3K, β-catenin, and VE-cadherin to NF-kB that activate angiogenesis were blocked significantly as indicated by western blot analysis. The expression of cell adhesion molecules of ICAM-1, VCAM-1, and E-selectin was inhibited by PLM-f74 as determined by ELISA. New anti-angiogenic and anti-cell adhesion materials suggesting high antioxidative capacities and reduced toxicities may be expected from marine resources.

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