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噬菌蛭弧菌的分离及初步鉴定

郑英珍[1,2]　汪建国[1]　李　明[3]　可小丽[1,2]　龚小宁[1]
(1. 中国科学院水生生物研究所,武汉　430072; 2. 中国科学院研究生院,北京　100049; 3. 武汉工业学院饲料科学系,武汉　430023)

摘要:采用双层平板法,以滤膜过滤的方法来收集噬菌蛭弧菌,以嗜水气单胞菌 (*Aeromonas hudrophila*)、荧光假单胞菌 (*Pseudomonas fluorescent*)和绿脓杆菌 (*Pseudomonas aeruginosa*)为宿主菌,进行噬菌蛭弧菌的分离研究;并在此基础上,通过接触酶检测和寄生性确认对噬菌蛭弧菌 (*Bdellovibrio bacteriovorus*)进行了初步的鉴定。结果表明未使用滤膜过滤,采用自来水琼脂双层平板法分离噬菌蛭弧菌的效果较好;并经过接触酶和寄生性检测初步鉴定此 BD-SP01菌株为噬菌蛭弧菌。

关键字:噬菌蛭弧菌;分离;鉴定
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噬菌蛭弧菌 (*Bdellovibrio bacteriovorus*)是一类体形较小,广泛分布于土壤、海水、污水等生境,运动极活泼的革兰氏阴性菌;具有“寄生”和“裂解 (溶菌)”宿主菌的生物学特性。这类生物最早是由 Stolp, *et al*. 在菜豆枯萎病假单胞菌体中发现[1]。它比通常的细菌小,因而能够侵入宿主细胞内裂解宿主。实验证明,蛭弧菌对致病菌的裂解能力明显大于非致病菌,且肠道病原菌更易被裂解[2]。此外,噬菌蛭弧菌在水体净化方面也可以发挥一定的作用[3]。近年来,随着养殖业的发展、养殖水体的污染,许多革兰氏阴性病原菌如嗜水气单胞菌等对水产动物造成了越来越严重的危害。鉴于蛭弧菌的这一特性,使得蛭弧菌能够成为水产养殖业中一种良好的微生态制剂。因此,越来越多的学者试图开发利用噬菌蛭弧菌作为水体微生态制剂,以净化水体并抑制病原[4]。本文主要研究了污水中噬菌蛭弧菌的分离及初步鉴定方法,以期为后续高效优质噬菌蛭弧菌的筛选和相应生物制剂的开发及进一步研究工作的开展奠定基础。

1　材料与方法

1.1　采样地点　将 6支干净的 15 mL旋盖离心管 121℃灭菌 20min。取武汉市龙王嘴污水处理厂污水 15 mL于灭菌的离心管中,编号,4℃保存。武汉市龙王嘴污水处理厂,位于武汉市武昌关山村龙王嘴。一号进水池,二号厌氧池,三号缺氧池,四号曝气池,五号出水池,六号初沉池。

1.2　宿主菌株　嗜水气单胞菌 (*Aeromonas hudrophila*)、荧光假单胞菌 (*Pseudomonas fluorescent*)、绿脓杆菌 (*Pseudomonas aeruginosa*)均为本实验室保存菌种。

1.3　培养基配置　双层平板法:上层采用自来水琼脂、1/10营养肉汤、1/500营养肉汤培养基 (含 0.8%琼脂),下层采用自来水琼脂、1/5营养肉汤培养基 (含 1.2%琼脂)。普通营养肉汤培养基 (NB)。培养基的 pH值调节到 7.0。121℃灭菌 20min后倾倒平板备用。

1.4　主要实验器材　25 mm滤器,25 mm混合纤维素滤膜:孔径分别为 3.0、1.2、0.8、0.22 μm。滤膜浸湿后放在滤器内,锡纸包裹,121℃灭菌 20min备用。

1.5　主要实验试剂　0.1%多聚赖氨酸 (Poly-L-Lysine Solution)、蛋清与甘油混合液 (1∶1)、2.5%戊二醛 (Glutaraldehyde)、3%过氧化氢 (H_2O_2)溶液、结晶紫混合液、碘液、脱色液、复染液[5]。

1.6　噬菌蛭弧菌的分离

1.6.1　宿主菌悬液的制备　宿主菌悬液的制备:将宿主菌接种于 NB固体平板上,30℃培养 24h,用 4 mL无菌水洗脱下来后 (每个平板的菌液收集到两个 EP管中),12000 r/min离心 2min,倒掉上清液,

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作者简介:郑英珍 (1983—),女,河北省唐山市人;博士研究生;主要从事鱼类病害方面的研究
通讯作者:汪建国,研究员,博士生导师; E-mail: wangjg@ihb.ac.cn

加入 1 mL无菌水,之后稀释三倍,4℃保存备用。

1.6.2 蛭弧菌的分离、纯化与培养 蛭弧菌的分离采用双层琼脂平板法,方法如下:取 1—6号样品各 2 mL分别用 3.0 μm滤膜过滤,收集滤液;依照以上方法收集经过 1.2 μm和 0.8 μm滤膜过滤后的滤液;取部分 0.8 μm滤膜过滤后滤液经 0.22 μm滤膜过滤后,弃滤液,取滤膜;收集 1—6号样品各 2 mL,与以上的滤液和滤膜一起备用。

分别取 200 μL滤液、100 μL宿主和 4 mL上层培养基(48℃保温)混匀后,铺于底层培养基上,30℃恒温培养,观察噬菌斑产生情况;取收集好的没有过滤的样品 200 μL、宿主 100 μL和 4 mL上层培养基混匀后,铺于底层培养基上,30℃恒温培养,观察噬菌斑的产生情况;取 0.22 μm滤膜与 100 μL宿主和 4 mL上层培养基混匀后,铺于底层培养基上,30℃恒温培养,观察噬菌斑产生情况。

经过 2—4d的培养后,平板上有透明、圆形、整齐的噬菌斑产生。挑取单个典型的噬菌斑经过 4次以上传代培养获得纯蛭弧菌株。

1.7 蛭弧菌的鉴定 根据《伯杰氏细菌鉴定手册》第八版中的分类及鉴定方法,对分离到的蛭弧菌进行下列方面的鉴定[6]。

1.7.1 接触酶鉴定 在干净的载玻片上滴一滴 3%过氧化氢溶液,将挖下来的噬菌斑倒置的放到过氧化氢溶液上,观察有无气泡产生。有为阳性,无为阴性[5]。

1.7.2 寄生性鉴定 在同一平板上挖取三个单斑,不加入宿主菌培养蛭弧菌,观察有无噬菌斑或菌落产生。若无则说明为专性寄生,若有则为非专性寄生。设立对照,噬菌斑取自同一平板,加入宿主培养,观察有无噬菌斑出现。设立三个平行。

1.7.3 革兰氏染色 挖取同一平板上的数个噬菌斑于 1.5 mL EP管中,加水 1 mL,浸泡 30min,使蛭弧菌充分的游离到水中。滴一滴菌液于干净的载波片上,风干后固定。用结晶紫混合液染 1min后,水洗;碘液作用 1min,水洗;用 95%乙醇溶液脱色,流滴至洗脱液至无色(约 30s);用复染液染 2—3min,水洗,风干。油镜观察,深紫色为革兰氏阳性细菌,红色为革兰氏阴性细菌。

1.7.4 扫描电镜观察 经过 4次以上传代获得的纯蛭弧菌株,挖取单斑于 1.5 mL EP管中,加入 20 μL灭菌水,浸泡 30min以上。取面积 1 cm ×1 cm的载玻片,将粘片剂均匀地涂在玻片上(先涂蛋清再涂多聚赖氨酸),待粘片剂将要干的时候把蛭弧菌液均匀的涂在玻片上,自然风干。在菌液将干时加 2.5%戊二醛于 4℃固定 2h,磷酸缓冲液清洗 3次,4℃锇酸固定 1h,磷酸缓冲液清洗 3次;脱水,50%、70%、80%、90%乙醇各 5—10min,100%乙醇脱水两次,每次 20min;醋酸异戊酯置换;临界点干燥;离子溅射仪(Eiko B-3 Ion Coater)镀膜喷金后,扫描电镜 Quanta 200(FEI, Holand)观察。

2 结 果

2.1 分离结果

采用双层平板法以绿脓杆菌、荧光假单胞菌和嗜水气单胞菌为宿主菌,通过过滤的收集方法检测污水水样发现,培养 3—4d后,未经过滤的四号曝气池水样在自来水平板(上下层均为自来水琼脂培养基)上形成大小相似,直径较小(0.1—0.25 cm),圆形,透明,边缘光滑,整齐,凹陷的噬菌斑(表 1)。挑选特征典型的噬菌斑进行传代,传代 4次之后得到一株噬菌蛭弧菌。记为 BD-SP01。BD-SP01对 3种宿主菌都有噬菌作用。

表 1 自来水双层平板上分离到的噬菌蛭弧菌

Tab.1 The isolated *Bdellovibrio bacteriovorus* in double layer plants of tap water

样品	未过滤样品			0.22 μm滤膜			0.8 μm滤液			1.2 μm滤液			3.0 μm滤液*		
	E	XS	56	E	XS	56	E	XS	56	E	XS	56	E	XS	56
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

注:1—6:样品编号;“+”表示分离得到了噬菌蛭弧菌;“-”表示未分离得到噬菌蛭弧菌;E:绿脓杆菌;XS:嗜水气单胞菌;56:荧光假单胞菌;*:未过滤样品即样品没有用滤膜过滤处理,0.22 μm滤膜即样品用滤膜处理后将滤膜按照滤液的方法进行培养,0.8、1.2、3.0 μm滤液即用相应的滤膜处理样品

Notes: 1—6: samples' number; "+": isolation of *Bdellovibro bacteriovorus*; "-": no isolation of *Bdellovibrio bacteriovorus*; E: *Pseudamonas aeruginosa*; XS: *Aeromonas hudrophila*; 56: *Pseudamonas fluorescent*; *: the samples were not filtered by filters, 0.22 μm filter membrane: incubated the used 0.22 μm filter membrane as filtered samples; 0.8, 1.2, 3.0 μm filtrate: used the corresponding filter membrane to filter the samples

其余分离方法分离到的菌株未纯化得到噬菌蛭弧菌。在分离的过程中,不同的培养基、不同的宿主菌、不同的水样以及不同的过滤孔径过滤水样的平板都有噬菌斑出现,只是在纯化传代时出现了菌株丢失的现象。

2.2 鉴定结果

接触酶鉴定　经检测,倒置的噬菌斑在过氧化氢溶液上有气泡产生,为接触酶阳性。

寄生性鉴定　所有无宿主菌的平板上既没有噬菌斑出现也没有菌落出现,有宿主菌的对照均有噬斑出现,说明寄生性检测为专性寄生。

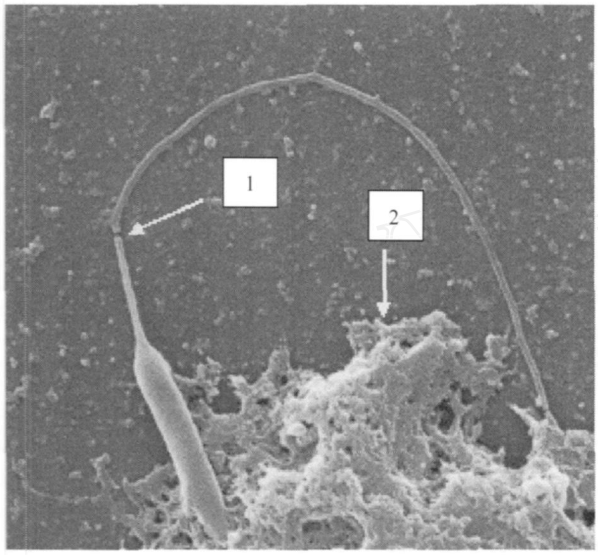

图1　污水处理厂分离到的
噬菌蛭弧菌(BD-SP01)(未过滤, 20000×)

Fig. 1　The *Bdellovibrio bacteriovorus* isolated from sewage disposal work (BD-SP01) (no filtration, 20000×)

1. 鞭毛及断裂点; 2. 蛋清杂质

1. flagellum and the breakpoint; 2. egg white impurity

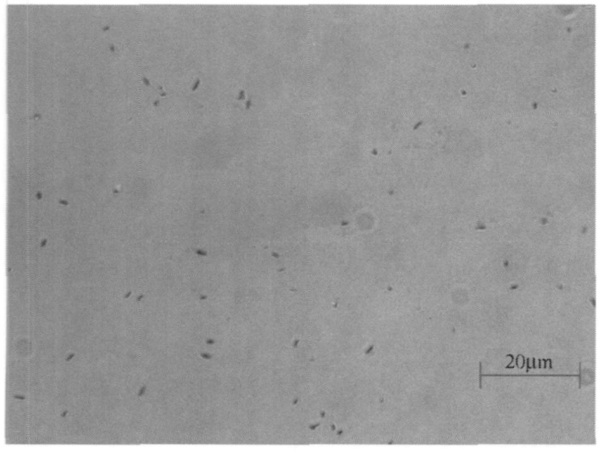

图2　BD-SP01 革兰氏染色(400×)

Fig. 2　The gram coloration of BD-SP01 (400×)

形态特征　分离到的蛭弧菌 BD-SP01 在扫描电镜下观察可见蛭弧菌呈杆状,单极鞭毛(图 1)。革兰氏染色阴性(呈红色),菌体大小一般为(0.2—0.5) μm ×(0.8—1.2) μm,也有长为 3.2 μm 的较长个体(图 2)。

通过接触酶和寄生性以及形态特征方面的鉴定,可以初步判断分离到的菌株为噬菌蛭弧菌。

3 讨　论

3.1 蛭弧菌的分离

培养基的营养成分过高造成宿主和杂菌生长快且多,使得蛭弧菌产生的噬菌斑被宿主覆盖,所以在分离噬菌蛭弧菌的时候要使用低营养成分的培养基。Stolp, *et al.*[7]早在 1963 年就提出了低营养的培养基用于分离噬菌蛭弧菌。而以后分离蛭弧菌也多用稀释了的培养基[8,9]。

不同孔径滤膜过滤后分离培养的蛭弧菌噬菌斑在数量上没有很大不同,孔径小的滤膜过滤后分离培养的蛭弧菌杂菌较少; 0.22 μm 滤膜分离培养的蛭弧菌与滤液分离培养的蛭弧菌噬菌斑在数量上没有明显的增多。

Varon 和 Shilo[10]使用低速离心(800g 5min)和高速离心(27000g 20min)法除去较大颗粒的杂质和较小的噬菌体,然后再用 1.2 μm 的滤膜过滤。同时过滤的步骤还可以由 Ficoll 梯度离心法代替。Jurkevitch E[8]应用 Varon 和 Shilo 的方法分离到了 30 株新的蛭弧菌。噬菌蛭弧菌的大小为 0.25—0.4 μm ×0.8 μm ×1.2 μm[11],在本次试验中应用微孔滤膜过滤的方法使蛭弧菌集中没有明显的效果。而蛭弧菌菌体宽 0.3 μm,比一般的细菌要小[9], 3.0、1.2、0.8 μm 滤膜过滤后采用双层平板法分离蛭弧菌,生成的噬菌斑并没有比未过滤的多,可见应用滤膜过滤的处理方法是使杂菌减少。

3.2 蛭弧菌的鉴定

根据《伯杰氏细菌鉴定手册》第八版中对噬菌蛭弧菌的分类及鉴定[4],噬菌蛭弧菌的 G+C 比例高(50.4 ±0.9 g分子%),接触酶阳性,对弧菌抑制剂 O/129 敏感,专性寄生或专性非寄生,蛋白酶活性低。而斯托普蛭弧菌(*Bdellovibrio stolpii*)的 G+C 比例低(42—43 g分子%),接触酶阳性,对弧菌抑制剂 O/129 敏感,兼性寄生或不寄生,蛋白酶活性高。斯塔尔蛭弧菌(*Bdellovibrio starrii*)的 G+C 比例低,接触酶阴性,对弧菌抑制剂 O/129 有抗性,兼性寄生或不寄生,蛋白酶活性中等,严格寄生于假单

胞菌。

噬菌蛭弧菌是专性寄生或专性非寄生细菌,而斯托普蛭弧菌和斯塔尔蛭弧菌均为兼性寄生或不寄生,即在无宿主菌存在的情况下也可以在营养培养基上以菌落的形式生长,通过检测寄生性即可知分离的菌株为哪种蛭弧菌。经检测寄生性结果为专性寄生,即为噬菌蛭弧菌。通过试验可知分离到的蛭弧菌可以寄生于荧光假单胞菌也可寄生于嗜水气单胞菌等其他细菌,则说明通过试验即可排除分离到的蛭弧菌是斯塔尔蛭弧菌的可能性。

经过电镜观察,分离到的蛭弧菌为杆状,端生单鞭毛。在扫描电镜样品处理的过程中,由于使用蛋清和多聚赖氨酸粘片,而蛋清因固定变性使得扫描的结果出现大量的杂质,蛋清变性之后结构变得膨大,很容易把蛭弧菌包裹进去,观察不到,造成损失。所以考虑到扫描的效果,在处理样品和粘片的时候尽量要用多聚赖氨酸,这样可以减少杂质的出现。

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THE ISOLATION AND THE ELEMENTARY IDENTIFICATION OF BDELLOVIBRIO BACTERIOVORUS

ZHENG Ying-Zhen[1,2], WANG Jian-Guo[1], LI Ming[3], KE Xiao-Li[1,2] and GONG Xiao-Ning[1]

(1. *Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan* 430072; 2. *Graduate School of the Chinese Academy of Sciences, Beijing* 100049; 3. *Feed Science Department, Wuhan Polytechnic University, Wuhan* 430023)

Abstract: *Bdellovibrio bacteriovorus* is a small gram-negative bacterium, and distributes over soil, freshwater, seawater and sewage. With regard to its great ability to lyse other gram-negative bacteria, the application of this microecological preparation to aquaculture is well expected. No identification, however, it has been given by other scholars previously. In the present study, we isolated the *B. bacteriovorus* and tried to confirm its identity.

Double layer plant was used to isolate *B. bacteriovorus* from sewage water. The sewage water was collected from six different pools of the sewage disposal p1ant of Wuhan, which were intake sump, anaerobic tank, anoxic pond, aeration tank, outlet sump and primary settling tank orderly. We did the identification of the bacterium for H_2O_2 activity and parasitical activity, and took the photo of the bacterium of SEM and the stain of gram after the isolation of the *B. bacteriovorus*.

In the isolation experiment, *Aeromonas hudrophila*, *Pseudomonas fluorescent* and *Vibrio anguillarum* were used as the host bacteria, and the six kinds of sewage water were filtered through 3.0 μm, 1.2 μm, 0.8 μm pore-size filters to collect the *B. bacteriovorus* individually. Then 100 μL host bacteria were added to 200 μL filtered water which mixed with 4mL molten top-layer culture medium, and were plated on bottom layer and incubated at 30℃. After the filtration through 0.8 μm filter, some portion of the left water passed through 0.22 μm filter. The filter membrane was mixed with 100μL host bacteria and the molten top layer culture medium plated on bottom layer and incubated at 30℃. The Bdellovobrio appeared as plaques, and then chosed one transparent and round plaque to purify for obtaining the pure Bdellovobrio after 2-4 days. We could obtain the pure *B. bacteriovorus* after 4 times purification. We isolated one strain of *B. bacteriovorus* from aeration tank with *Pseudomonas fluorescent* as host bacterium using the tap water plant of two layers. And the sewage water was not filtrated with any filter membrane.

Then we did the elementary identification of H_2O_2 activity and parasitical activity. Three percents H_2O_2 solution was dropped on the microscope slide and one plaque was cut out from top layer and immerged in the solution with the invertion side. If bladder appeared, the bacterium should be masculine of H_2O_2; if not, the bacterium should be feminine of H_2O_2. On the other hand, the isolated bacterium with no host bacteria was incubated at 30℃ to test its parasitical characteristic. If there was no bacterium grown on the plant, the isolated bacterium was obligate parasitical bacterium. If not, the isolated bacterium was not the obligate parasitical bacterium. The two identification results showed that this bacterium was masculine of H_2O_2 activity and parasitical activity.

Some plaques were cut out from the one plant to a 1.5mL EP cannulation and dipped in 1mL water for 30min to dissociate the *B. bacteriovorus* from the plaque. After that, one blob of the dissociated bacterium was dropped on the microscope slide and air-dried the blob, then fixed. The bacterium on the microscope slide was stained as the following step: firstly, stained the bacterium with crystal purple for 1min and wash; secondly, stained with iodine solution for 1min and wash; thirdly, decolored with 95% ethanol for 30s; fourthly, stained with safranine solution for 2-3min, washed and air-dried. Finally, it was observed by oil lens. The result of this identification proved the isolated bacterium was negative of Gram.

Besides, the SEM photo of this *B. bacteriovorus* had been taken. The photo showed that the bacterium was rod shaped and had one flagellum on one side. The size of the bacterium was 2.0 μm.

It was substantiated that the isolated bacterium was Bdellovobrio bacteriovorus.

Key words: *Bdellovibrio bacteriovorus*; Isolation; Elementary identification