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Research Paper 研究报告

红斑丹毒丝菌免疫原性蛋白的鉴定及其编码基因的克隆和测序

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摘要：【目的】为深入研究红斑丹毒丝菌的免疫保护性抗原及其致病机制，采用免疫蛋白组学技术鉴定红斑丹毒丝菌的免疫原性蛋白。【方法】通过SDS-PAGE电泳分离红斑丹毒丝菌C43065株的NaOH提取抗原，用兔抗NaOH提取抗原抗血清经Western blot检测免疫原性蛋白，通过MALDI-TOF质谱技术鉴定蛋白种类，并对部分免疫原性蛋白的编码基因进行克隆和测序。【结果】通过MALDI-TOF质谱技术从C43065株NaOH提取抗原中鉴定出9个免疫原性蛋白，分别为SpaA、伴侣蛋白GroEL、烯醇化酶、ATP结合盒转运蛋白、丙酮酸脱氢酶复合物E1、甘油醛-3-磷酸脱氢酶、果糖二磷酸醛缩酶、50S核糖体蛋白L1、30S核糖体蛋白S4。其中烯醇化酶、ATP结合盒转运蛋白、甘油醛-3-磷酸脱氢酶和果糖二磷酸醛缩酶已被证实与链球菌、牙龈卟啉单胞菌、脑膜炎奈瑟菌和结核分枝杆菌的致病性相关。C43065株伴侣蛋白GroEL、烯醇化酶、ATP结合盒转运蛋白、丙酮酸脱氢酶复合物E1、甘油醛-3-磷酸脱氢酶和果糖二磷酸醛缩酶编码基因大小分别为1614、1296、1260、1005和867 bp，与已公布的红斑丹毒丝菌Fujisawa株相应基因的相似度高达98%。【结论】本文所鉴定的9个免疫原性蛋白，为进一步开展红斑丹毒丝菌保护性抗原及其致病机制研究奠定基础。

关键词：红斑丹毒丝菌，NaOH提取抗原，免疫原性蛋白，质谱，基因克隆

丹毒丝菌(*Erysipelothrix*)根据DNA-DNA杂交和PCR分为红斑丹毒丝菌(*E. rhusiopathiae*)和扁桃体丹毒丝菌(*E. tonsillarum*)，前者有1a、1b、2、4、5、6、8、9、11、12、15、16、17、19、21和N等16种血清型，后者有3、7、10、14、20、22和23等7种血清型[1−2]。其中红斑丹毒丝菌是猪丹毒、禽类败血症、人类丹毒的致病菌，并能引起鱼类脏器出血和肾脏肿大等疾病，给养殖业造成巨大的经济损失，也给人们的食品卫生和人体健康带来威胁[3]。临床研究表明，大约75%以上的

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猪源分离菌株属于1型和2型红斑丹毒丝菌，而其余血清型的红斑丹毒丝菌在猪败血症、荨麻疹、关节炎、淋巴结炎和心内膜炎中偶尔出现[4–6]。目前，在国内外用1型和2型红斑丹毒丝菌研发猪丹毒的灭活疫苗和活菌疫苗，而这些疫苗只能预防特急性、急性和风疹性疾病，但无法预防该菌引起的慢性疾病，且在疫苗应用中仍有疫情再次爆发，至今世界各地尚未彻底消灭该疾病[7–9]。因此，研究红斑丹毒丝菌的免疫原性蛋白，可为猪丹毒的预防和治疗提供理论依据。

Kitajima等[10]检测了2型红斑丹毒丝菌NaOH提取抗原疫苗的保护作用，结果表明该疫苗能保护SPF猪受同源菌株和异源菌株的致死性感染，且疫苗组分中的64–67 kDa蛋白均可与攻毒前免疫血清和康复期免疫血清发生特异性反应，表明这些蛋白可能具有免疫保护作用。Makino等[11]用单抗从2型红斑丹毒丝菌基因组文库中克隆出64 kDa保护性抗原蛋白的编码基因，结果表明其C端有“GW”二肽起始的8个串联重复序列，与肺炎链球菌胆碱结合蛋白C端重复序列有高度同源性[12]，而该蛋白通过其C端重复序列能够与红斑丹毒丝菌、枯草芽孢杆菌和肺炎链球菌等革兰氏阳性菌细胞壁磷脂壁酸结合，因此将其命名为表面保护性抗原A (surface protective antigen A，SpaA)。后来Shimoji等[13]用原核表达系统分别表达2型红斑丹毒丝菌的重组SpaA和其C端重复序列(SpaA-C)并检测保护作用，结果表明SpaA免疫组小鼠和SpaA-C免疫组小鼠对同源菌株攻毒的保护率分别为100%和20%。刘丹丹等[14]比较了2型红斑丹毒丝菌重组SpaA和其N端342个氨基酸序列(SpaA-N)和SpaA-C的保护作用、结果显示SpaA免疫组小鼠和SpaA-N免疫组小鼠对同源菌株攻毒的保护率均为100%，但SpaA-C免疫组小鼠攻毒后全部死亡。后来Borrathybay等[15]构建了2型红斑丹毒丝菌*SpaA*基因的敲除株，经甲醛处理分别制备野生株和敲除株的灭活疫苗，保护试验结果显示野生株灭活疫苗和敲除株灭活疫苗对小鼠的保护率分别为80%和40%。Shi等[16]检测了2型红斑丹毒丝菌重组胆碱结合蛋白B(choline-binding protein B，CbpB)的保护作用，结果表明CbpB免疫组小鼠对同源菌株攻毒的保护率为80%，而免疫电镜观察结果显示该蛋白定位于细胞表面。上述研究结果表明，SpaA和CbpB是红斑丹毒丝菌表面的主要保护性抗原，可作为研制猪丹毒亚单位疫苗的候选抗原。本文用SDS-PAGE电泳技术分离2型红斑丹毒丝菌NaOH提取抗原，Western blot检测NaOH提取抗原中的免疫原性蛋白，MALDI-TOF质谱鉴定免疫原性蛋白，并对部分免疫原性蛋白基因进行克隆和测序。

1 材料和方法

1.1 材料

1.1.1 菌株和质粒：2型红斑丹毒丝菌C43065株购自国家兽医微生物菌种保藏中心；大肠杆菌DH5α和pMD18-T载体购自大连TaKaRa公司。

1.1.2 主要试剂和实验动物：Brain Heart Infusion (BHI)液体培养基和BHI固体培养基购自Bacto公司；DL2000 DNA marker和Protein molecular weight marker (Broad)购自大连TaKaRa公司；兔抗重组SpaA抗血清由本实验室保存[14]；HRP标记的羊抗兔IgG和邻苯二胺(DAB)购自上海生工生物工程公司；X-Gal为北京鼎国公司产品；IPTG为Promega公司产品；清洁级新西兰白兔购自湖南斯莱克景达实验动物有限公司。

1.2 C43065株NaOH提取抗原的制备

将红斑丹毒丝菌C43065株接种于含有0.1% Tween 80的BHI (BHI-T)固体培养基37 °C培养24 h，将单菌落接种于BHI-T液体培养基37 °C培养18 h，离心收集菌体，利用NaOH提取法[10]制备

C43065株的NaOH提取抗原样本，用Bradford法测定其蛋白含量。

1.3 免疫血清的制备

用NaOH提取抗原对清洁级兔进行3次皮下注射，免疫剂量为200 μg，初次免疫与弗氏完全佐剂等计量混合，第2次和第3次免疫分别将NaOH提取抗原与弗氏不完全佐剂等计量混合，第3次免疫1周后从颈动脉采血，分离血清，–20 ℃保存。

1.4 免疫血清抗体效价的间接ELISA测定

以NaOH提取抗原为包被抗原，利用刘丹丹等[14]的方法检测兔抗NaOH提取抗原免疫血清的抗体效价。即用50 mmol/L碳酸盐缓冲液(pH 9.6)制备蛋白含量为200 μg/mL NaOH提取抗原溶液，将100 μL抗原溶液加入96孔ELISA板孔内，4 ℃包被过夜，用含0.05% Tween 20的PBS (PBS-T20)缓冲液洗涤3次。用含1%脱脂奶粉和1% BSA的PBS-T20缓冲液于37 ℃封闭3 h，将待测血清用封闭液进行稀释后加入ELISA板孔内，于37 ℃孵育2 h，用PBS-T20缓冲液洗涤3次。将HRP标记的山羊抗兔IgG用封闭液进行1:1000稀释，加入ELISA板孔中，37 ℃孵育2 h。用PBS-T20缓冲液洗涤3次，将含0.04% OPD的磷酸盐柠檬酸缓冲液(pH 4.8)加入ELISA板孔内，37 ℃避光反应30 min，用2 mol/L H_2SO_4溶液终止反应后，用酶标仪测定OD_{492}的吸光值。ELISA结果表明免疫血清的抗体效价为1:1200，其*OD*值为1.23。

1.5 免疫原性蛋白的Western blot检测

通过SDS-PAGE电泳分离C43065株NaOH提取抗原后，用半干式电转仪将蛋白条带转移至硝酸纤维素膜上，用5%脱脂奶粉溶液37 ℃封闭过夜，与兔抗NaOH提取抗原抗血清(1:1200)室温孵育2 h，然后与HRP标记的羊抗兔IgG (1:1000)室温孵育1 h，每步完成后严格洗膜，最后加DAB显色液显色。

1.6 免疫原性蛋白的MALDI-TOF质谱鉴定

用Western blot检测NaOH提取抗原中的免疫原性蛋白，用手术刀从凝胶上切取免疫原性蛋白条带。按照Shevechenko等[17]的方法对胶条进行漂洗，脱色、脱水、干燥、胰蛋白酶消化、萃取处理后在384孔质谱靶上点样，采用4700型飞行时间质谱仪(MALDI-TOF/TOF-MS，Applied Biosystems, Foster City，CA，USA)鉴定蛋白种类。采用4000 Series Explorer™ software version 3.0软件采集样品；从800 Da到4000 Da采集一级谱图，采用interpreation从高到低采集6个母离子。使用GPS工作站(GPS Explorer™ version 3.6，Mascot version 2.1) 搜索NCBI的非冗余蛋白质数据库。设置可变修饰Carboxymethyl(C)、Oxidation(M)，搜索母离子偏差设为0.1 Da，MS/MS片段偏差设为0.1 Da。蛋白与离子的置信区均高于95%，且至少有两个肽段得分大于50或一个肽段得分大于60，确认蛋白。

1.7 部分免疫原性蛋白编码基因的克隆和测序

根据红斑丹毒丝菌Fujisawa株基因组(Accession No. AP012027)中的编码伴侣蛋白GroEL、磷酸丙酮酸水合酶(又称烯醇化酶)、ATP结合盒转运蛋白、甘油醛-3-磷酸脱氢酶和果糖二磷酸醛缩酶的基因序列。用Primer Premier 5.0软件设计5对引物(表1)，引物由上海生工生物工程有限公司合成。用5对引物经PCR从C43065株基因组DNA中分别扩增出编码伴侣蛋白GroEL、烯醇化酶，ATP结合转运蛋白、甘油醛-3-磷酸脱氢酶和果糖二磷酸醛缩酶的基因序列。PCR产物经胶回收试剂盒纯化后与pDM18-T连接，转化大肠杆菌DH5α，菌落PCR初选含有重组质粒的白色菌落，用试剂盒提取质粒DNA后进行PCR鉴定。结果为阳性者送往上海生工生物工程公司进行测序，对测序结果进行分析。

表1. 引物的序列、位置及扩增片段长度

Table 1. Sequences and locations of the primers and length of expected fragments

Primers	Nucleotide sequences (5′→3′)	Primer positions[a]	Size of fragments/bp
GroEL-F	ATGGCTAAAGATGTACGTTATGGAC	1339539–1339563	1614
GroEL-R	TTAATACATTGGCGGCATTTCAG	1341152–1341130	
Eno-F	ATGCCAAGTATTATTGATATTTATG	1395504–1395528	1296
Eno-R	TTATTTTTTGATGTTGAAGAA	1396799–1396779	
ABC-F	ATGGGAAATATTAAAAAGATTTTAG	455653–455677	1260
ABC-R	TTATTGCCAGATTGTTTGTAAGGAA	456912–456888	
GapC-F	ATGACAGTTAAAGTAGCAATTAACG	1618401–1618425	1005
GapC-R	TTAGAATTTTGAAGCAACGTAATG	1619405–1619382	
FBA-F	ATGGCATTAGTATCAGCTAAAGGCA	1709942–1709966	867
FBA-R	TTAAGCTTGGTTAGCTGAACCCATC	1710808–1710784	

[a] Position relative to the genomic sequences of *E. rhusopathiae* strain Fujisawa deposited in GenBank (AP012027).

2 结果和分析

2.1 C43065株NaOH提取抗原的制备及其免疫原性检测

SDS-PAGE结果显示，C43065株NaOH提取抗原含有分子量约为68、64、58、47、46、41、35、30、25、23 kDa的蛋白条带，编号分别为B18、B19、B20、B21、B22、B23、B24、C1、C2和C3(图1-A)。Western blot结果显示，C43065株NaOH提取抗原中的10条蛋白与兔抗NaOH提取抗原抗血清发生反应(图1-B泳道1)，其中64 kDa蛋白与抗重组SpaA抗体发生特异性反应(图1-B泳道2)，表明C43065株NaOH提取抗原中的10条蛋白均具有不同程度的免疫原性。

2.2 免疫源性蛋白的MALDI-TOF质谱鉴定

将C43065株NaOH提取抗原中的11个免疫原性蛋白条带切取后，胶内酶解并用于质谱鉴定。成功鉴定了免疫原性蛋白B19、B20、B21、B22、B23、B24、C1、C2和C3(表2)，它们分别为SpaA、伴侣蛋白GroEL (chaperone protein GroEL)、烯醇化酶(enolase)、ATP结合盒转运蛋白(ATP-binding cassette transporter)、丙酮酸脱氢酶复合物E1 (Pyruvate dehydrogenase complex E1)、甘油醛-3-磷酸脱氢酶(Glyceraldehyde-3-

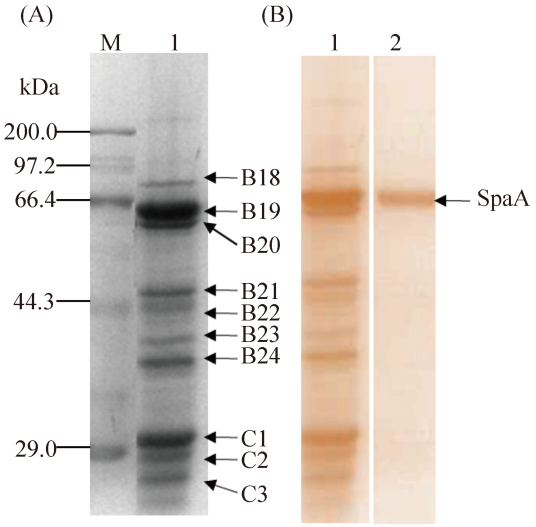

图 1. 红斑丹毒丝菌C43065株NaOH提取抗原及其免疫原性的SDS-PAGE和Western blot检测

Figure 1. SDS-PAGE and Western blot analysis of the immunogenic proteins in the NaOH-extracted antigen of *E. rhusiopathiae* C43065. A: M, Protein marker; 1, NaOH-extracted antigen. B: 1, NaOH-extracted antigen reacted with rabbit antiserum against the NaOH-extracted antigen; 2, NaOH-extracted antigen reacted with rabbit antiserum against the recombinant SpaA.

表2. 红斑丹毒丝菌菌体表面免疫原性蛋白的MALDI-TOF/TOF质谱鉴定

Table 2. Identification of the surface immunogenic proteins of *E. rhusiopathiae* C43065 by MALDI-TOF/TOF Mass Spectrometry

Band	Protein name	Accession No.	Protein MW /kDa	Protein score	Protein score CI%	Total Ione score
B19	Surface protective antigen A	gi|5881766	68	305	100	200
B20	Chaperone protein GroEL	gi|259505371	59	360	100	186
B21	Phosphopyruvate hydratase	gi|259505317	47	185	100	100
B22	ATP-binding cassette transporter	gi|259504519	46	89	98.558	72
B23	Pyruvate dehydrogenase complex E1	gi|259504500	41	137	100	45
B24	Glyceraldehyde-3-phosphate dehydrogenase	gi|259505103	38	197	100	112
C1	Fructose-bisphosphate aldolase class-II	gi|259505006	32	339	100	252
C2	50S ribosomal protein L1	gi|259505063	25	105	99.962	51
C3	30S ribosomal protein S4	gi|259504432	22	405	100	305

phosphate dehydrogenase)、果糖二磷酸醛缩酶(Fructose-bisphosphate aldolase class-II)、50S核糖体蛋白L1 (50S ribosomal protein L1)和30S核糖体蛋白S4 (30S ribosomal protein S4)(表2)。但不能鉴定免疫原性蛋白B18(图1)。表明红斑丹毒丝菌NaOH提取抗原含有与细菌基础代谢相关的组成型蛋白。

2.3 部分免疫源性蛋白编码基因的克隆和测序

琼脂糖凝胶电泳结果显示，用引物对GroEL-F/GroEL-R、Eno-F/Eno-R、ABC-F/ABC-R、GapC-F/GapC-R和FBA-F/FBA-R (表1)经PCR从C43065株基因组DNA中分别扩增出大小约为1.6、1.3、1.2、1.0和0.9 kb的DNA片段(省略图)，与预期值相符。将PCR产物连接至pMD18-T载体上，转化大肠杆菌DH5α，通过PCR鉴定重组质粒DNA后送上海生工生物工程公司进行测序。DNA测序结果表明，编码C43065株伴侣蛋白GroEL、烯醇化酶(Eno)、ATP结合盒转运蛋白(ABCt)、甘油醛-3-磷酸脱氢酶(GapC)和果糖二磷酸醛缩酶(FBA)基因大小分别为1614、1296、1260、1005和867 bp，GenBank登录号分别为KF980880、KF980881、KF980882、KF980883和KF980884。BLAST分析结果表明，红斑丹毒丝菌C43065株和已公布Fujisawa株(登录号为AP012027) *eno*、*ABCt*、*gapC*和*fba*基因核苷酸序列的相似性均为99%，而C43065株和Fujisawa株*groEL*基因序列的相似性为98%。

3 讨论

Kitajima等[10]的研究表明，红斑丹毒丝菌NaOH提取抗原疫苗具有交叉保护作用，发现疫苗组分中的64–67 kDa蛋白具有免疫原性，但是这些免疫原性蛋白的种类和生物学功能尚不清楚。因此，本文用免疫蛋白组学技术从红斑丹毒丝菌C43065株NaOH提取抗原组分中鉴定出SpaA、伴侣蛋白GroEL、烯醇化酶、ABC转运蛋白、丙酮酸脱氢酶复合物E1、甘油醛-3-磷酸脱氢酶和果糖二磷酸醛缩酶等免疫原性蛋白，并对编码伴侣蛋白GroEL、烯醇化酶、ABC转运蛋白、丙酮酸脱氢酶复合物E1、甘油醛-3-磷酸脱氢酶和果糖二磷酸醛缩酶基因进行克隆和测序。

GroEL在新生蛋白质的正确折叠和组装以及热或化学逆境下变性蛋白质的复性中起重要作用，同时在细菌的跨膜转运及插入细胞质膜方面

都起重要作用，它也在细菌对噬菌体的防御过程中发挥重要的作用[18–19]。Feng等[20]的研究表明，烯醇化酶是猪链球菌细胞表面的保护性抗原，同时也在猪链球菌对宿主细胞黏附、定殖和侵染过程中发挥重要作用。Basavanna等[21]构建肺炎链球菌ABC转运蛋白基因的突变株并检测其致病性，结果表明与野生株相比突变株对小鼠的致病性明显降低。Maeda等[22]的研究表明，甘油醛-3-磷酸脱氢酶是牙龈卟啉单胞菌(*Porphyromonas gingivalis*)菌毛的辅助粘附素，他们推测本菌靠甘油醛-3-磷酸脱氢酶的粘附作用定植于牙周组织并导致牙周炎。Tunio等[23]构建脑膜炎奈瑟菌(*Neisseria meningitidis*)编码甘油醛-3-磷酸脱氢酶基因的突变株，黏附试验结果表明与野生株相比突变株对人上皮细胞和内皮细胞的黏附能力明显降低。Saber等[24]的研究表明，果糖二磷酸醛缩酶是结核分枝杆菌的葡萄糖代谢酶，它具有良好的保护作用。Shi等[16]发现红斑丹毒丝菌ATCC 19414株胞外分泌性蛋白样本含有侣蛋白GroEL、丙酮酸脱氢酶复合物E1、甘油醛-3-磷酸脱氢酶和果糖二磷酸醛缩酶等组成型蛋白，而免疫电镜观察结果显示丙酮酸脱氢酶复合物E1和甘油醛-3-磷酸脱氢酶定位于细胞表面。上述研究结果表明，伴侣蛋白GroEL、烯醇化酶、ABC转运蛋白、甘油醛-3-磷酸脱氢酶和果糖二磷酸醛缩酶与大肠杆菌、链球菌、牙龈卟啉单胞菌、脑膜炎奈瑟菌和结核分枝杆菌的致病性相关。但是伴侣蛋白GroEL、烯醇化酶、ABC转运蛋白、甘油醛-3-磷酸脱氢酶和果糖二磷酸醛缩酶在红斑丹毒丝菌致病过程的作用尚不清楚。所以，本文从红斑丹毒丝菌基因组DNA中克隆出编码伴侣蛋白GroEL、烯醇化酶、ABC转运蛋白、甘油醛-3-磷酸脱氢酶和果糖二磷酸醛缩酶的全基因序列，为红斑丹毒丝菌致病机理研究奠定基础。

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Identifying immunogenic proteins of *Erysipelothrix rhusiopathiae* C43065 by MALDI-TOF mass spectrometry and cloning their encoding genes

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Abstract: [**Objective**] To identify immunogenic proteins of *Erysipelothrix rhusiopathiae* C43065. [**Methods**] Antigens were extracted from *E. rhusiopathiae* C43065 by the alkaline extraction method. Proteins in the NaOH-extracted antigen were separated by SDS-PAGE and transferred to nitrocellulose membranes, and then Western blotting was performed with rabbit antiserum against the NaOH-extracted antigens. The immunogenic protein bands were identified by MALDI-TOF mass spectrometry. The genes encoding 5 major immunogenic proteins was amplified by PCR from the genomic DNA of *E. rhusiopathiae* C43065, and inserted into the pMD18-T vector and then sequenced. [**Results**] A total of 9 immunogenic surface proteins in the NaOH-extracted antigen from *E. rhusiopathiae* C43065 were successfully identified by MALDI-TOF mass spectrometry. Four of the proteins were putative virulence-associated proteins: enolase, ATP-binding cassette transporter, glyceraldehyde-3-phosphate dehydrogenase and fructose-bisphosphate aldolase class-II. The genes encoding the chaperone protein GroEL, enolase, ATP-binding cassette transporter, glyceraldehyde-3-phosphate dehydrogenase and fructose-bisphosphate aldolase class-II were 1614, 1296, 1260, 1005 and 867 bp in length, and the nucleotide sequences homologies of the genes between the C43065 strain and the previously reported *E. rhusiopathiae* Fujisawa strain was more than 98%. [**Conclusion**] Several putative virulence-associated proteins in the NaOH-extracted antigen of *E. rhusiopathiae* C43065 will be useful for elucidating the roles of these proteins in the pathogenesis of the organism.

Keywords: *Erysipelothrix rhusiopathiae*, NaOH-extracted antigen, immunogenic proteins, mass spectrometry, gene cloning

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