学校编码：10384　　分类号____密级____

学号：21620141152542　　UDC____

厦门大学

硕 士 学 位 论 文

大肠杆菌中二硫键形成相关基因改造和导入糖基化作用的基因编辑及其用于全长人源工程抗体的表达

Gene Editing on Bacterial Genome Associated with Improvement of Disulfide Bonding and Glycosylation Implantation and Its Application on Expression of Full-length Engineering Huamn Antibody

李 琼

指导教师姓名：李少伟 教 授

专 业 名 称：生物化学与分子生物学

论文提交日期：2017 年 4 月

论文答辩时间：2017 年 5 月

学位授予日期：2017 年 6 月

答辩委员会主席：

评　阅　人：

2017 年 4 月

厦门大学学位论文原创性声明

本人呈交的学位论文是本人在导师指导下,独立完成的研究成果。本人在论文写作中参考其他个人或集体已经发表的研究成果，均在文中以适当方式明确标明，并符合法律规范和《厦门大学研究生学术活动规范（试行）》。

另外，该学位论文为（　　）课题（组）的研究成果，获得（　　）课题（组）经费或实验室的资助，在（　　）实验室完成。（请在以上括号内填写课题或课题组负责人或实验室名称，未有此项声明内容的，可以不作特别声明。）

声明人（签名）：

年　　月　　日

厦门大学学位论文著作权使用声明

本人同意厦门大学根据《中华人民共和国学位条例暂行实施办法》等规定保留和使用此学位论文，并向主管部门或其指定机构送交学位论文（包括纸质版和电子版），允许学位论文进入厦门大学图书馆及其数据库被查阅、借阅。本人同意厦门大学将学位论文加入全国博士、硕士学位论文共建单位数据库进行检索，将学位论文的标题和摘要汇编出版，采用影印、缩印或者其它方式合理复制学位论文。

本学位论文属于：

（　　）1.经厦门大学保密委员会审查核定的保密学位论文，于　　年　　月　　日解密，解密后适用上述授权。

（　　）2.不保密，适用上述授权。

（请在以上相应括号内打“√”或填上相应内容。保密学位论文应是已经厦门大学保密委员会审定过的学位论文，未经厦门大学保密委员会审定的学位论文均为公开学位论文。此声明栏不填写的，默认为公开学位论文，均适用上述授权。）

声明人（签名）：

年　　月　　日

目 录

Table of contents

摘要

近年来基因工程抗体成为现代生物学及医学领域的研究热点，具有不可估量的市场前景。如何经济高效地表达具有活性的抗体分子，成为基因工程抗体研究中亟需解决的问题。目前 CHO 表达系统发展较成熟，在抗体表达中应用广泛，然而该系统培养要求精细、操作复杂、容易污染，具有较高的生产成本。*E.coli* 表达系统具有遗传背景清楚、生长繁殖快、表达量高、工艺简单、节约成本等诸多优点。但是全长抗体分子需要依靠二硫键正确配对形成完整的结构，而 *E.coli* 细胞质内的高度还原状态使其应用于抗体表达时无法形成稳定的二硫键，不能进行正确的氧化折叠。此外，*E.coli* 缺乏有效的糖基化系统，不能对抗体进行糖基化修饰。因此基于大肠杆菌表达系统，构建一种可促进二硫键正确配对，且能够对外源蛋白进行糖基化修饰的新型表达系统具有重要的意义。

Red 同源重组系统利用 Exo、Beta、Gam 3 种同源重组酶以及含有短同源臂的线性 DNA 打靶片段，通过简单的操作即可在 *E.coli* 基因组上实现精确的基因敲除或敲入。CRISPR-Cas9 基因编辑系统是近年来迅速发展并得到广泛应用的一种高效基因编辑系统，该系统靶点分布频率高、设计简单、操作简单，可在原核及真核细胞基因组的任何位置进行高效、特异、多种形式的基因编辑。

本研究旨在对 *E.coli* 基因组进行基因改造，获得一种可在细胞质中形成二硫键的表达菌株；利用该菌株进行全长抗体表达并验证其性质；在可促进细胞质中二硫键形成菌株的基础上，进行 *E.coli* 糖基化系统构建的初步尝试。

首先，利用 Red 同源重组系统，先后敲除编码硫氧还蛋白还原酶的 *trxB* 基因及编码谷胱甘肽还原酶的 *gor* 基因；利用 CRISPR-Cas9 基因编辑系统，将去掉信号肽的 *DsbC* 编码区及 T7 表达调控元件整合至 *E.coli* 基因组，在细胞质中过表达具有分子伴侣活性的二硫键异构酶 DsbC，使细胞质呈高度还原状态并促进二硫键正确配对，获得了可在细胞质中形成二硫键的 *E.coli* 蛋白表达菌株 TGD。

其次，以含有双表达框的 pET-duet 为载体，构建了两种抗乙肝病毒表面抗原的抗体 23E7 和 HUE6F6 的轻重链共表达质粒；利用本研究构建的菌株 TGD、NEB 公司可促进细胞质中二硫键形成的菌株 SHuffle 以及本研究出发菌株 ER2566，分别进行抗体 23E7 和 HUE6F6 的表达；三种抗体表达菌株菌体形态

相似，生长状况无明显差异；表达所得抗体经 protein A 亲和层析后达到较高纯度；抗体 23E7 在 TGD 菌株中表达量为 SHuffle 和 ER2566 菌株的 6.4 倍，抗体 HUE6F6 在 TGD 菌株中的表达量为 SHuffle 和 ER2566 菌株的 4.1 倍，TGD 菌株中的两种抗体的表达量明显提高。

第三，利用 SDS-PAGE、Western blot、分子排阻色谱、分析型超速离心、抗原结合活性评价、病毒中和活性评价等多种方式，对 TGD 菌株、CHO 细胞、SHuffle 菌株及 ER2566 菌株表达的两种抗体进行了性质检测与比较。与 SHuffle 和 ER2566 菌株相比，TGD 菌株表达的抗体可形成四聚体形式的完整抗体，组分单一，纯度较高，抗原结合活性及中和活性均强于 SHuffle 和 ER2566 菌株表达的抗体。

最后，利用 Red 同源重组系统，对 *E.coli* 中编码 O-抗原连接酶的 *waaL* 基因进行敲除；将空肠弯曲菌中编码寡糖转移酶的 *pglB* 基因构建至 pTO-T7 载体，使其在 *E.coli* 中表达；利用该糖基化改造菌株表达的抗体可检测到明显的寡糖信号，可能含有初步的糖基化修饰，为 *E.coli* 中 N-连接糖基化系统的建立提供了参考。

综上所述，本研究构建的细胞质呈氧化状态且能促进二硫键形成的 *E.coli* 菌株可高效表达全长抗体，为利用 *E.coli* 进行全长抗体表达的相关研究奠定了基础；空肠弯曲菌 N-连接糖基化系统的引入为 *E.coli* 中糖基化系统的建立提供参考，有望构建出一种既可促进二硫键正确配对又能进行糖基化修饰的新型原核表达系统。

关键词：大肠杆菌；基因编辑；氧化还原环境；抗体表达；糖基化

Abstract

In recent years, genetically engineered antibodies have been widely used in scientific research and clinical practice, and has a very broad market prospects. How to express active antibodies efficiently and economically becomes a growing problem. At present, the Chinese hamster ovary (CHO) cell expression system is already quite mature and has been widely used in antibody expression. Nevertheless, this system is very time consuming, complicated to operate, easy to pollute and high cost of goods relative to prokaryotic expression system. *E.coli* prokaryotic expression system is the most attractive expression system, it has many advantages such as clear genetic background, fast growth and reproduction, high yields, ease of use, relatively low cost and so on. The full-length antibodies need to rely on disulfide bonds to form a complete structure, and need be modified by glycosylation to function. However, the cytoplasm of *E.coli* is the highly reduced state and can not form a stable disulfide bond. In addition, *E.coli* lacks an effective glycosylation system and can not glycosylate the antibody.Thus, antibodies are misfolded, poorly expressed, and has low activity when expressed in the cytoplasm of *E.coli*. Therefore, this is of great significance to constructing a novel expression system which can promote the correct pairing of disulfide bonds and be able to glycosylate the exogenous protein based on *E.coli* prokaryotic expression system.

The Red mediated recombination requires the participation of three different homologous recombinases of Exo, Beta, Gam and linear DNA targeting fragments containing short homologous arms, this system can be used to delete or insert DNA sequences on the *E.coli* genome accurately. The high frequency of Targeted point, simple design and operation, various forms, high specificity and efficiency of CRISPR-Cas9 make it be the first selected technology for gene editing at any position of prokaryotic and eukaryotic cell genome in recent years.

The aim of this study is to genetically edit the *E.coli* genome to obtain a protein expression strain that could form disulfide bonds in the cytoplasm. Full-length antibodies were expressed by the strain, their propertie were determined and compared to other protein expression strains. Further more, an initial attempt for construction of glycosylation system in *E.coli* was made on the base of above researchs.

Firstly, the *trxB* gene encoding the thioredoxin reductase and the *gor* gene encoding glutathione reductase were knocked out by Red homologous recombination system. The CRSPR-Cas9 gene editing system was used to integarte the coding region of DsbC containing the T7 regulatory element into the *E.coli* genome to overexpress the disulfide isomerase DsbC with chaperone activity in the cytoplasm. We obtained an novel *E.coli* protein expression strain named TGD, whose cytoplasm was highly oxidized and it could form stable disulfide bonds.

Secondly, we constructed two kinds of light chain and heavy chain co-expression plasmid of hepatitis B virus surface antibody 23E7 and HUE6F6, the pET-duet containing double multiple cloning sites was used as the vector. Antibodies expression was performed using the TGD strain constructed in this study, the SHuffle strain saled by NEB company which can correctly folding disulfide bonded proteins in its cytoplasm,and the recipient ER2566 strains respectively. The cell morphology and the growth status of the three strains was similar. The purity of expressed antibodies was highly purified after purified by affinity chromatography, The expression level of antibody 23E7 in TGD strain was 6.4 times that of SHuffle and ER2566 strains, the expression level of antibody HUE6F6 in TGD strain was 4.1 times higher than that of SHuffle and ER2566 strain, the expression of two antibodies in TGD strain was significantly improved.

Thirdly, the properties of antibodies expressed by three strains were tested and compared by SDS-PAGE, Western blot, molecular exclusion chromatography, analytical ultracentrifugation, antigen-binding activity evaluation and virus neutralization activity. Compared with SHuffle and ER2566 strains, The antibodies expressed by the TGD strain can form tetramer, components are simple, with high purity, antigen binding activity and neutralizing activity were stronger than those expressed in SHuffle and ER2566 strains. The properties of antibodies expressed by TGD strains had obvious advantages.

Finally, the *waaL* gene encoding O-antigen ligase in *E.coli* was knocked out using the Red homologous recombination system. The *pglB* gene encoding oligosaccharide transferase in *Campylobacter jejuni* was constructed into the pTO-T7 vector, and the plasmid was transformed into *E.coli* for expression. Antibodies expressed by glycosylated strain can detect the obvious oligosaccharide signal, may contain a preliminary glycosylation modification, which provides a reference for the establishment of an N-linked glycosylation system in *E. coli*.