Appl Microbiol Biotechnol (2016) 100:8693–8699
DOI 10.1007/s00253-016-7829-5

MINI-REVIEW

Genetic engineering of probiotic *Escherichia coli* Nissle 1917 for clinical application

Bingming Ou[1,2] · **Ying Yang**[1,2] · **Wai Liang Tham**[1,2,3] · **Lin Chen**[1,2,4] · **Jitao Guo**[5] · **Guoqiang Zhu**[1,2]

Received: 13 July 2016 / Revised: 31 August 2016 / Accepted: 3 September 2016 / Published online: 17 September 2016
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Abstract *Escherichia coli* strain Nissle 1917 (EcN) has been used as a probiotic. Genetic engineering has enhanced the utility of EcN in several vaccine and pharmaceutical preparations. We discuss in this mini review the genetics and physical properties of EcN. We also discuss the numerous genetic engineering strategies employed for EcN-based vaccine development, including recombinant plasmid transfer, genetic engineering of cryptic plasmids or the EcN chromosome, EcN bacterial ghosts and its outer membrane vesicles. We also provide a current update on the progress and the challenges regarding the use of EcN in vaccine development.

Keywords Nissle 1917 · Genetic modification · Cryptic plasmids · Chromosome · Clinical application

Bingming Ou and Ying Yang are equally contributing lead authors.

✉ Guoqiang Zhu
yzgqzhu@yzu.edu.cn

[1] College of Veterinary Medicine, Yangzhou University, Yangzhou 225009, China

[2] Jiangsu Co-Innovation Center for Important Animal Infectious Diseases and Zoonoses, Yangzhou 225009, China

[3] Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver V6T1Z4, Canada

[4] Jiangsu Agri-animal Husbandry Vocational College, Taizhou 225300, China

[5] Key Laboratory of Pathogenic Microbiology and Immunology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China

Introduction

Escherichia coli Nissle 1917 (EcN) is a non-pathogenic *E. coli* strain that confers health benefits without known harmful effects on various hosts and is marketed under the trade name Mutaflor (Sonnenborn and Schulze 2009). First isolated by Dr. Alfred Nissle during World War I, EcN has proven to be effective at competing with other enteropathogens and has been used as a treatment for diarrhea and other diseases (Nissle 1918; Nissle 1925; Sonnenborn and Schulze 2009). EcN colonizes the gut of various hosts, including humans and animals such as rats and pigs (Monteiro et al. 2009; Nissle 1925). EcN also antagonizes intestinal pathogens in vivo (Sonnenborn and Schulze 2009). EcN appears to be a safe therapeutic agent and does not produce any enterotoxins or cytotoxins associated with pathogenic *E. coli* strains (Sonnenborn and Schulze 2009). EcN has recently been studied for its potential utility in vaccine development, primarily in inducing immune responses (Baxter 2007). Traditionally, attenuated bacteria or viruses are used as vaccines to present essential antigens to the immune system to initiate protective immune responses (Baxter 2007). These approaches could theoretically be enhanced by using genetically modified probiotics (Sonnenborn and Schulze 2009). EcN is also widely used in diagnostics and drug development. EcN possesses tumor-specific colonization properties in mice (Stritzker et al. 2007; Zhang et al. 2012) and has thus been used to diagnose solid tumors in clinical studies (Brader et al. 2008). EcN can activate multiple pro-drugs in situ (Lehouritis et al. 2015), showing that the combined use of EcN and pro-drugs is promising for solid tumor treatments. Recent studies have tried to engineer EcN to enhance its ability to induce immune responses and to improve its utility in diagnostic and

therapeutic applications (Table 1). Here, we review aspects of EcN genetics and the numerous engineering approaches that have been used with this probiotic strain.

EcN genetics

EcN possesses two small cryptic plasmids, termed pMUT1 and pMUT2. Both plasmids, genetically stable and non-transferable, are completely sequenced and only found in *E. coli* Nissle 1917 (Blum-Oehler et al. 2003; Sonnenborn and Schulze 2009). The pMUT1 carries a ColE1-type-replication system and pMUT2 contains a ColE2-like replication system and another mobilization system; however, no other open reading frames with known functions have been identified in both plasmids (Oswald 2006). The stability of these two cryptic plasmids has been utilized as a framework from which to express exogenous genes in EcN. A plasmid-free variant of *E. coli* strain DSM 6601(EcN) has been constructed and was observed not to be functionally different from the wild-type EcN strain (Blum-Oehler et al. 2003; Oswald 2006; Sonnenborn and Schulze 2009). However, these cryptic plasmids may serve a role in defense against bacteriophage predation (Feldgarden et al. 1995). The most commonly used laboratory strain is EcNc which is cured of both cryptic plasmids (Schlee et al. 2007) and has been used for vaccine development (Remer et al. 2009; Seo et al. 2012). This plasmid-free variant can also be used as a live vector for recombinant plasmids based on the plasmids pMUT1 and pMUT2 (Oswald 2006).

The stability of the EcN chromosome has been tested both through 100 sequential passages in vitro and in an in vivo experiment where passage of EcN through the intestinal tract of 14 newborn children for 24 months did not show any changes in the sequence (Sonnenborn and Schulze 2009). EcN does not take up plasmids of the IncFI and IncFII types, which often contain virulence factors (conjugation frequency 0 %) (Sonnenborn and Schulze 2009). Moreover, phage-encoded genetic information for the production of enterohemorrhagic *E. coli* (EHEC) Shiga-like toxins is not taken up by EcN (Datz et al. 1996; Magistrelli et al. 1992; S. O. 1997; Smith et al. 1983; Sonnenborn and Schulze 2009). In addition, there are several genomic islands in the EcN chromosome, which synthesize "fitness factors" crucial to its ability to colonize a host (Hacker and Carniel 2001). However, the *pks* island, which encodes machinery for the synthesis of a hybrid peptide-polyketide genotoxin, colibactin (McCarthy et al. 2015), that induces DNA damage and genomic instability and gene mutations in mammalian cells (Cuevas-Ramos et al. 2010), was identified in the EcN genome (Nougayrede et al. 2006; Olier et al. 2012), which raises the issue of a potential safety liability risk involved in the long-term use of EcN.

Recombinant EcN strains

Several recombinant vectors have been used to modify EcN for clinical applications. The B-subunit of the *E. coli* heat-labile enterotoxin (LT-B) was co-expressed with peptides harboring T-cell epitopes of the *Yersinia enterocolitica* heat-shock protein 60 (Y-hsp60) in AIDA (adhesin-involved-in-diffuse-adherence) autotransporter system vectors (Benz and Schmidt 2011; Li et al. 2008; Niewerth et al. 2001) and introduced into EcN, leading to successful epitope presentation of the LT-B as well as the functional T-cell epitopes of Y-hsp60 on the surface of EcN. Glucagon-like peptide 1 (GLP-1) and the pancreatic and duodenal homeobox protein (PDX-1) were expressed from a modified flagellar type III secretion vector under the control of the *fliC* promoter (Majander et al. 2005) and introduced into EcN (Duan et al. 2008). These recombinant EcN strains secreted GLP-1 and PDX-1, leading to cellular insulin secretion in response to glucose (Duan et al. 2008). EcN was also modified to express the *V. cholerae* autoinducer 1 (CAI-1) (Duan and March 2008). Recombinant EcN-*cqsA* inhibited the expression of cholera toxin (CT) and the toxin co-regulated pilus (TCP) in both monocultures of *V. cholerae* and co-cultures of epithelial cells and *V. cholerae* (Duan and March 2008). Human α-defensin 5 (HD5) and β-defensin 2 (HBD2) have also been expressed from EcN (Seo et al. 2012) and HBD2 was shown to have antimicrobial activity (Seo et al. 2012). EcN was used to express interleukin 10 (IL-10) and had efficacy in a mouse model of inflammatory bowel disease (IBD) (Gardlik et al. 2012).

The tumor-targeting properties of EcN were employed to inhibit mouse B16 melanoma and 4T1 breast tumors by expressing the azurin protein in EcN (Zhang et al. 2012). B16 melanoma and orthotopic 4T1 breast tumor growth were markedly inhibited, and pulmonary metastasis was prevented in immunocompetent mice, without significant toxicity (Zhang et al. 2012). EcN has also been modified to express omega-3 fatty acids, including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which may have beneficial effects on human health (Amiri-Jami et al. 2015).

Genetic engineering of EcN cryptic plasmids

Stable cloning vectors for the probiotic *E. coli* strain DSM6601 were developed through the integration of cassettes encoding antibiotic resistance proteins and fluorescent proteins into the cryptic plasmids pMUT1 and pMUT2 (Oswald 2006). pMUT1 has also been modified with the AIDA system carrying the coding sequences for *ospA*/*ospG* of *B. burgdorferi* for the purpose of immunizing mice with recombinant EcN strains to generate IgG antibody titers against OspA and OspG (Buddenborg et al. 2008; Buddenborg 2005). pMUT2 was engineered to express the enterotoxigenic *E. coli*

Table 1 Current overview of genetically modified Nissle1917 for clinical exploration

Application category	Disease/function	Origin	Animal model/cell line	Administration/ application	Reference
Preventative	Diarrhea in piglets and newborn calves	ETEC	Mice	Oral	(Remer et al. 2009; Zhang 2010)
	V. cholera infection	*V. cholerae*	Caco-2 epithelial cells & Infant mouse	Oral	(Duan and March 2008; Duan and March 2010)
	Yersiniosis	*Y. enterocolitica*	Antigen presenting cells & T-cells	Oral	(Konieczny et al. 2000)
	AIDS	HIV	4 to 8-week-old CD-1 mice	Oral/rectal	(Rao et al. 2005)
	Influenza	Human influenza virus, H1N1 influenza virus	Antigen presenting cells & T-cells	Oral	(Konieczny et al. 2000; Rosenthal 2014)
	Lyme disease	*Borrelia burgdorferi*	BALB/c mice	Intragastral	(Buddenborg et al. 2008)
	Ocular surface diseases	NM	HCjE cell line & conjunctival guinea pig epithelial cells	Topical administration	(Stein et al. 2013)
	Trachoma	Ct	HcjEcells, BALB/cmice & guinea pigs	Conj/s.c. route	(Inic-Kanada et al. 2015; Montanaro et al. 2015)
	Peanut allergy	*Arachis hypogaea*	BALB/c mice	s.c. injection	(Rosenthal 2014)
Therapeutic	IBD	NM	C57BL/6 mice & pigs	Gastric gavage & Oral gavage	(Gardlik et al. 2012; Whelan et al. 2014)
	CD	NM	No	Oral	(Seo et al. 2012)
	B16 melanoma & 4T1 breast tumors	NM	Female BALB/c and C57BL/6 mice	i.v. injection	(Zhang et al. 2012)
	Diabetes	ND	Caco-2 cells	ND	(Duan et al. 2008)
	Peanut allergy	*A. hypogaea*	BALB/c mice	s.c. injection	(Rosenthal 2014)
Carriers	Invade mammalian cells	No	B16 cell and HCT-116 cell	Tumor target treatment	(Bai et al. 2015)
	EcN OMV platform	No	BALB/c mice	s.c. injection	(Rosenthal 2014)
Diagnostic	Liver metastasis	NM	rodent models	Orally administered diagnostic	(Danino et al. 2015)
Health care	Human health	Omega 3 fatty acids	No	NM	(Amiri-Jami et al. 2015)

ND not determined, *NM* not mentioned, *i.v.* intravenously injected, *s.c.* subcutaneously injected, *conj* conjunctival immunization, *IBD* inflammatory bowel disease, *ETEC* Enterotoxigenic *Escherichia coli*, *CD* Cancer Diseases, *AIDS* Acquired Immune Deficiency Syndrome *HIV* human immunodeficiency virus, *Ct Chlamydia trachomatis*, *HCjE*, Human conjunctival epithelial, *B16* murine melanoma B16 cell line, *HCT-116* human colon cancer cell line

Table 2 Summary of genetically modified parts in *E.coli* Nissle1917 for clinical exploration

GM parts in EcN	Specific site in EcN	Antigens/beneficial molecules	GM methods/mechanisms/ vectors	Reference
Cytoplasmic transformation	ND	Autoinducer molecule CAI-1	pCAI-1	(Duan and March 2008)
	ND	T-cell epitopes of Y-hsp60/ HA epitope	AIDA autotransporter system plasmid	(Konieczny et al. 2000)
	ND	FedF adhesin	pnirBMisL	(Zhang 2010)
	ND	IL10	pMEC	(Gardlik et al. 2012)
	ND	HD5/HBD2	pAR1219 & a recombinant pET-28a(+)	(Seo et al. 2012)
	ND	Azurin protein	pSUM vector	(Zhang et al. 2012)
	ND	GLP-1 & PDX-1	FliC secretion tag (Majander et al. 2005)	(Duan et al. 2008)
	ND	EPA/DHA	pfBS-PS	(Amiri-Jami et al. 2015)
Cryptic plasmids	pMUT1/pMUT2	Tc-Red or Kn-Gfp	NM	(Oswald 2006)
	pMUT2	K88 fimbrial adhesin	NM	(Remer et al. 2009)
	pMUT1	OspA/OspG	AIDA autotransporter system	(Buddenborg et al. 2008)
	pMUT1	AvCys	Hemolysin A secretion system	(Whelan et al. 2014)
Chromosome	Downstream of the fliC promoter region	CAI-1	One-step inactivation (Datsenko and Wanner 2000)	(Duan and March 2010)
	NM	Amino acids 114–162 ofgp41	pVDL9.3 (Tzschaschel et al. 1996) & pEHLYA2-SD (Fernández et al. 2000)	(Rao et al. 2005)
	Random integration in chromosome	Azurin protein	Transposons plasmid pR6K-Tps-cm-inv-hly	(Bai et al. 2015)
	16S locus in EcN chromosome	pTKW106alp7A luxCDABE cassette	hok/sok maintenance system (Wood et al. 1990; Wu and Wood 1994) & p16Slux plasmid (Riedel et al. 2007)	(Danino et al. 2015)
Shell structure	EcN BGs	MOMPs, PmpC & N-PmpC	pBGKB & pGLysivb	(Inic-Kanada et al. 2015; Montanaro et al. 2015; Stein et al. 2013)
	EcN OMV	Hemagglutinin N-terminal subunit, peanut allergen Arah2	Keio collection deletions (Baba et al. 2006)	(Rosenthal 2014)

GM genetic modified, *ND* not determined, *NM* not mentioned, *AIDA* adhesin-involved-in-diffuse-adherence, *Y-hsp60* Yersinia enterocolitica heat-shock protein 60, *HD5* human α-defensin 5, *HBD2* humanβ-defensin 2, *GLP-1* glucagon-like peptide 1, *PDX-1* pancreatic and duodenal homeobox gene 1, *CAI-1* cholera autoinducer 1, *MOMPs* major outer membrane proteins, *PmpC* polymorphic membrane protein C, *Ct Chlamydia trachomatis*, *AvCys* cystatin from the rodent nematode *Acanthocheilonema viteae*, *N-PmpC* N-terminal portion (amino acid 1–893) of the chlamydial PmpC

K88 fimbrial adhesin for expression on the EcN surface (Remer et al. 2009). pMUT1 was modified to express nematode immunomodulator cystatin from *Acanthocheilonema viteae* (AvCys) (Whelan et al. 2014). The transgenic probiotic EcN-AvCys significantly decreased intestinal inflammation in murine acute colitis, and high doses of the EcN-AvCys were well tolerated by post-weaning piglets (Whelan et al. 2014).

Genetic modification of the EcN chromosome

The stable inheritance of engineered functions is essential for strains to be used as live vaccines. Several approaches have been used to modify the EcN chromosome. A genetically engineered EcN strain that expressed a portion of the Gp41 HIV protein secreted micromolar levels of anti-HIV peptides and colonized mice for long periods of time (Rao et al. 2005). A genetically modified EcN with insertion of cholera autoinducer 1 gene *cqsA* in the downstream of the *fliC* promoter region in the chromosome showed a good prophylaxis and treatment effect in the infant mice models infected with *V. cholera* (Duan and March 2010). EcN mutants lacking portions of the flagellin hypervariable region are able to colonize the BALB/c mice intestinal tract, suggesting that EcN could be used for flagella display applications (Yang et al. 2016). EcN modified to co-express invasin (*inv* gene from *Yersinia pseudotuberculosis*) and hemolysin (*hly* locus from *Listeria monocytogenes*) became invasive to mammalian cells (Bai et al. 2015) and may have applications in gene therapy. EcN was also used to develop an orally administered diagnostic that can non-invasively indicate the presence of liver metastasis by producing easily detectable signals in urine (Danino

et al. 2015). The luxCDABE cassette was integrated into the 16S locus of the EcN chromosome to generate a luminescent signal. This strain was then used to generate a high-contrast urine signal through selective expansion in liver metastases and high expression of a *lacZ* reporter. After oral delivery, the engineered EcN colonized tumors in rodent models of liver metastasis, but did not colonize healthy organs or fibrotic liver tissue (Danino et al. 2015).

EcN bacterial ghosts (BGs) and outer membrane vesicles (OMVs)

BGs are considered to be hollow sac-like structures that can be modified to express foreign proteins or be filled with drugs (Kudela et al. 2011; Paukner et al. 2006; Mayr et al. 2005; Muhammad et al. 2012). EcN BGs have been developed to target ocular surface diseases (Stein et al. 2013) and were found to be non-toxic to guinea pig conjunctival cells (Stein et al. 2013). EcN BGs have been used to express major outer membrane proteins (MOMPs) and the polymorphic membrane protein C (PmpC) from *Chlamydia trachomatis* (Montanaro et al. 2015). The two foreign chlamydial antigens were retained after processing to EcN BGs, and attachment to conjunctival epithelial cells did not reduce cell viability (Montanaro et al. 2015). Modifications of this approach have shown efficacy in conjunctiva immunization approaches designed to reduce inflammation in guinea pigs challenged with *C. trachomatis* (Inic-Kanada et al. 2015). These studies represent important steps in constructing delivery systems based on EcN BGs that are suitable for treating eye diseases.

Modified EcN strains have been used for OMV production platforms, including those for a subunit of the H1N1 influenza virus hemagglutinin (Rosenthal 2014). Immunization of BALB/c mice with these OMVs increased IFN-γ:IL-4T-cell secretion and enhanced cross-protection against H3N2 challenge (Rosenthal 2014). Similar approaches were used to express the peanut allergen Arah2 to reduce anaphylaxis in a mouse model of peanut allergy sensitization (Rosenthal 2014). This study demonstrated EcN OMV platform could be a promising host of PLP (pathogen-like particles) vaccines.

Conclusions

This review summarized several ways in which *E. coli* Nissle1917 has been genetically modified over the past two decades (Table 2). It appears that EcN is a versatile probiotic that can be adopted for various clinical applications. However, potential health risks remain, including sepsis (Gronbach et al. 2010; Guenther et al. 2010) and DNA damage (Cuevas-Ramos et al. 2010; Nougayrede et al. 2006). EcN also loses its probiotic properties when introduced from the basolateral side of epithelia (Sabharwal et al. 2016), as could happen in the case of IBD due to compromised barriers. The long-term safety of using genetically modified EcN strains should be considered.

Acknowledgments The authors are grateful for grants from the Chinese National Science Foundation (Grant No. 30571374, 30771603, 31072136, 31270171) and the Genetically Modified Organisms Technology Major Project of China (2014ZX08006-001B), a project founded by the Jiangsu Overseas Research & Training Program for University Prominent Young and Middle–aged Teachers and Presidents and the China Postdoctoral Science Foundation (2013 M541740) and Jiangsu innovation research program for University postgraduate (KYLX15_1382).

Compliance with ethical standards

Conflicts of interest The authors declare that they have no competing interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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