Article

Biotechnology and Bioengineering

Nisin-Inducible Secretion of a Biologically Active Single-Chain Insulin Analog by *Lactococcus lactis* NZ9000

Daphne T.W. Ng,[1] Casim A. Sarkar[1,2]

[1]Department of Bioengineering, University of Pennsylvania, Philadelphia, Pennsylvania

[2]Department of Chemical and Biomolecular Engineering, University of Pennsylvania, 240 Skirkanich Hall, 210 S. 33rd Street, Philadelphia, PA 19104-6321; telephone: 215-573-4072; fax: 215-573-2071; e-mail: casarkar@seas.upenn.edu

Received 1 November 2010; revision received 25 January 2011; accepted 3 March 2011

Published online 9 March 2011 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/bit.23130

ABSTRACT: Oral delivery of insulin to diabetic patients is highly desirable because it would be non-invasive and more closely mimic normal physiology, but this route of administration typically results in low bioavailability due to low pH and enzymatic degradation along the gastrointestinal tract. To explore an alternative approach that may mitigate these obstacles and also facilitate local synthesis of new therapeutic protein molecules in the small intestine, we engineered the food-grade bacterium *Lactococcus lactis* (NZ9000) for nisin-inducible expression and secretion of a bioactive single-chain insulin (SCI) analog, SCI-57. We show that the addition of nisin during early-log phase has a modest inhibitory effect on cell growth but induction during mid-log phase has a negligible impact on proliferation, suggesting a tradeoff between cell growth rate and duration of induction. We find that a signal peptide such as *usp45* is necessary for secretion of SCI-57 into the medium; furthermore, we demonstrate that this secreted SCI-57 is biologically active, as assessed by the ability of conditioned *L. lactis* medium to stimulate Akt signaling in differentiated 3T3-L1 adipocytes. Finally, we show that the biological activity of SCI-57 was enhanced by near-neutral or slightly alkaline pH during induction, which is comparable to the pH in the small intestine, and by removal of a C-terminal purification tag. This study demonstrates that food-grade bacteria can be engineered to secrete bioactive insulin analogs and opens up the possibility of oral insulin delivery using live microorganisms.

Biotechnol. Bioeng. 2011;108: 1987–1996.

© 2011 Wiley Periodicals, Inc.

KEYWORDS: *Lactococcus lactis*; single-chain insulin; oral protein delivery

Correspondence to: Casim A. Sarkar

Contract grant sponsor: American Diabetes Association
Contract grant number: 7–09-IN-24
Contract grant sponsor: National Science Foundation CAREER Award
Contract grant number: 1055231
Contract grant sponsor: University of Pennsylvania
Additional supporting information may be found in the online version of this article.

Introduction

Oral administration is one of the most convenient ways of delivering drugs, but therapeutic proteins often must be administered by an invasive method such as intravenous or subcutaneous injection (Morishita and Peppas, 2006). Oral delivery of protein drugs is not generally feasible due to poor stability during passage through the gastrointestinal tract and low permeability across the intestinal wall, resulting in insufficient bioavailability. To increase oral bioavailability of proteins, various encapsulation strategies have been developed to protect the polypeptides from enzymatic digestion (Muller, 2010), but most have encountered roadblocks that prevent them from advancing to a clinical setting (Khafagy el et al., 2007). Thus, better oral delivery systems are needed and one promising option is the microorganism *Lactococcus lactis*.

L. lactis is a Gram-positive bacterium widely used in the food industry for production of fermented products such as buttermilk and cheese and is therefore routinely consumed in these foods. *L. lactis* has a safe association with humans and has been proposed for use as a probiotic (Balcazar et al., 2007). There has been increasing interest in the use of *L. lactis* as a mucosal delivery vehicle because it can survive passage through the stomach acid and contact with bile (Klijn et al., 1995) and it can be engineered to express and secrete targeting molecules and adjuvants (Nouaille et al., 2003). In this host, antigens and DNA have been introduced for mucosal vaccine delivery, single-chain variable fragments (scFvs) for anti-infectives, and allergens for allergy prevention (Wells and Mercenier, 2008). To address potential safety concerns of using live *L. lactis* in humans, the thymidylate synthase gene can be removed from the host genome, rendering the auxotrophic bacteria dependent on thymidine or thymine for survival and thus biologically contained (Steidler et al., 2003). In addition, since the recombinant protein is still locally produced when the

© 2011 Wiley Periodicals, Inc.

bacteria reach the intestine, proteolytic degradation is attenuated. Recently, a study involving the use of interleukin-10-secreting *L. lactis* to treat Crohn's disease has passed phase I clinical trials, supporting the notion that this live microorganism is a viable platform for oral protein delivery (Braat et al., 2006; Steidler et al., 2000). Here, we evaluated whether *Lactococcus lactis* would be capable of producing a bioactive insulin analog, since this would open the door for using this host as a vehicle for oral insulin delivery.

In addition to clear benefits in the ease of administration and improved patient compliance, oral delivery of insulin also most accurately recapitulates normal physiological delivery. After absorption in the intestine, orally delivered insulin reaches the portal system, more closely approximating what occurs in a non-diabetic individual (Still, 2002). Insulin is normally secreted by pancreatic β-cells in the form of a single-chain precursor, proinsulin, which is subsequently cleaved into separate A (21 residues) and B (30 residues) chains (Hua, 2010). However, this heterodimeric protein with three disulfide bonds (two interchain and one intrachain) would be challenging to synthesize using *L. lactis*. We therefore chose to heterologously express a single-chain insulin analog, SCI-57, which contains four substitutions in the A and B chains (one of which is present in Novalog® and another in Humalog®, rapid-acting insulin analogs already in clinical use by injection) and a 6-residue linker (GGGPRR) connecting these two chains, resulting in a single polypeptide 57 amino acids in length (Hua et al., 2008). SCI-57 not only resembles the folding and biological activity of wild-type insulin, but it also has enhanced thermodynamic stability and reduced aggregation, and allows for simpler single-chain synthesis, making it an attractive insulin analog for oral delivery (Hua et al., 2008; Rajpal et al., 2009).

We inducibly expressed SCI-57 in *L. lactis* strain NZ9000 using the NICE system, which is based on a two-component signaling system involved in the biosynthesis of the bacteriocin nisin (Mierau and Kleerebezem, 2005). Briefly, upon nisin-binding, NisK, a histidine kinase, autophosphorylates and transfers its phosphate group to NisR, which when activated induces transcription of a gene of interest under the control of promoter P*nisA* (de Ruyter et al., 1996b; Kuipers et al., 1998). Previous studies of this system using a reporter gene show a linear dose–response curve for increasing amounts of nisin (de Ruyter et al., 1996a).

Here, we demonstrate that, upon nisin induction, *L. lactis* can secrete SCI-57 if the gene is fused to the *usp45* secretion signal (van Asseldonk et al., 1990), although bacterial growth rate depends on the timing of nisin induction. We further demonstrate that the secreted SCI-57 is biologically active by assaying for insulin receptor signaling in an adipocyte cell line. Interestingly, total SCI-57 expression does not correlate with bioactivity on cells, suggesting that proper folding of the polypeptide is a bottleneck in maximizing functional yield. We identify culture pH as an important regulator of bioactivity and show that a neutral to slightly alkaline pH can significantly enhance the fraction of functional secreted SCI-57. Finally, we demonstrate that the biological activity of this analog can be even more greatly enhanced by removing a C-terminal RGS-His tag, which would be necessary anyway before proceeding to in vivo studies.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions

Bacterial strains and plasmids used in this work are listed in Table I. *Escherichia coli* was grown in TY medium (8 g tryptone, 5 g yeast extract, and 5 g sodium chloride per liter) at 37°C with shaking and *L. lactis* was grown in M17 medium (Oxoid, Hamshire, UK) containing 0.5% glucose (GM17) at 30°C statically (i.e., without shaking). Solid media were prepared by adding agar (15 g/L) to the corresponding broth. Chloramphenicol (Cm) was used at a final concentration of 10 μg/mL when culturing *E. coli* EC1000 or *L. lactis* NZ9000 harboring pNZPnisA:CYTO-LLO, pNZPnisA:SCI-57his, pNZPnisA:uspSCI-57his, or pNZPnisA:uspSCI-57.

DNA Manipulations and Transformations

Plasmid DNA from *E. coli* was isolated with a Qiagen miniprep kit (Qiagen, Valencia, CA) following the standard procedure; plasmid DNA from *L. lactis* was isolated with the same protocol except for an additional incubation of the cells with 4 mg/mL lysozyme (USB Affymetrix, Cleveland, OH) in P1 buffer at 37°C for 30 min. Phusion high-fidelity DNA polymerase (NEB, Ipswich, MA) was used in all PCR reactions as recommended by the manufacturer. Restriction enzymes and T4 DNA ligase were purchased from NEB. DNA was transformed into *E. coli* as described previously (Pope and Kent, 1996). DNA was transformed into *L. lactis* by electroporation as described in the manufacturer's manual (MoBiTec, Göttingen, Germany).

Primers used for DNA amplification are listed in Table II. The gene encoding the single-chain insulin analog SCI-57 was constructed from primers 10/12 by extension PCR. Primers 11/12 were then used to amplify this SCI-57 template and the resulting product was cut with *Nco*I and *Eco*RI for ligation into the similarly cut pRDV (Binz et al., 2004), giving rise to pRDV:SCI-57. SCI-57 was PCR amplified from pRDV:SCI-57 using primers 1/3 and 2/3 in a preliminary step for building full constructs with and without *usp45* leader sequence, respectively. The resulting PCR products were further amplified with primers 4/9 and 2/9, respectively, to add the *usp45* signal and RGS-His tag or only the RGS-His tag. The P*nisA* promoter region was PCR amplified using primers 7/8,

Table I. Bacterial strains and plasmids used.

	Characteristics	Sources
Strains		
E. coli EC1000	RepA^{+} MC1000, Km^{r}, carrying a single copy of the pWV01 *repA* gene in *glgB*	Leenhouts et al., 1996
L. lactis NZ9000	*L. lactis* MG1363 (*nisRK* genes on the chromosome)	Kuipers et al., 1998
Plasmids		
pRDV:SCI-57	pRDV containing the SCI-57 gene; Amp^{r}	This work
pNZPnisA:CYTO-LLO	Modified pNZ8048 containing P*nisA* promoter with downstream His-tagged *hly* gene; Cm^{r}	Bahey-El-Din et al., 2008
pNZPnisA:SCI-57his	Modified pNZ8048 containing P*nisA* promoter with downstream RGS-His-tagged *SCI-57* gene; Cm^{r}	This work
pNZPnisA:uspSCI-57his	Modified pNZ8048 containing P*nisA* promoter with downstream RGS-His-tagged *SCI-57* gene; fusion with *usp45* secretion leader; Cm^{r}	This work
pNZPnisA:uspSCI-57	Modified pNZ8048 containing P*nisA* promoter with downstream *SCI-57* gene without tag; fusion with *usp45* secretion leader; Cm^{r}	This work

with 8 introducing an *Eco*RI site at the end of P*nisA*. The resulting product was fused to the SCI-57-RGS-His gene product with or without *usp45* leader by assembly PCR. The assembled products were gel-purified and sequentially digested with *Kpn*I and *Bgl*II. The digested products were then ligated into the similarly digested pNZPnisA:CYTO-LLO (plasmid courtesy of Dr. Cormac Gahan, University College Cork) using T4 DNA ligase. The ligation mixture was transformed into chemically competent *E. coli* EC1000 (strain courtesy of Dr. Jan Kok, University of Groningen). After confirmation of the clones by DNA sequencing, the plasmids (pNZPnisA:uspSCI-57his and pNZPnisA:SCI-57; Fig. 1) were transformed into electrocompetent *L. lactis* NZ9000.

To remove the RGS-His tag from pNZPnisA:uspSCI-57his to obtain pNZPnisA:uspSCI-57, primers 5/6 were used to PCR amplify from pRDV:SCI-57. The resulting SCI-57 gene product without the RGS-His tag was sequentially digested using *Kpn*I and *Bsp*EI and the product was ligated into the similarly digested pNZPnisA:uspSCI-57his vector, giving rise to pNZPnisA:uspSCI-57 (Fig. 1).

Table II. Primers used in this study.

Primer number	Primer name	Nucleotide sequence (5′–3′)
1	SCI57_f_usp	TCCGGAGTTTACGCTTTCGTTAACCAGCAC
2	SCI57_f_nousp	CACTCAAAGAATTCATGTTCGTTAACCAGCAC
3	SCI57_r_rgshis	TGGTGGTGATGGTGGGATCCTCTGTTGCAGTAGTTTTCCA
4	usp45_f	GCACTCAAAGAATTCATGAAAAAAAAGATTATCTCAGCTATTTTAATGTCTACA GTGATACTTTCTGCTGCAGCCCCGTTGTCCGGAGTTTACGCT
5	uspSCI57_f	CCCCGTTGTCCGGAGTTTACGCTTTCGTTAACCAGCAC
6	stopSCI57_r_KpnI	GAACTAGTGGTACCTCATTAGTTGCAGTAGTTTTCC
7	PnisA_f_BglII	TACAGCTCCAAGATCTAGTC
8	PnisA_r_EcoRI	CATGAATTCTTTGAGTGCCTCCTTATA
9	rgshis_r_KpnI	GAACTAGTGGTACCTCATTAATGATGGTGGTGATGGTGG
10	SCI-57gene_f	ATATATCCATGGGCTTCGTTAAC CAGCACCTGTGCGGTTCTGACCTGGTTGAAGCTCTGTACCTGGTTTGCGGTGAACGT GGTTTCTTCTACACCGACCCGACCGGTGGTGGTCCGCGTCGTGGTATCGTTGAACAGTGCTG CCACTCTATCTGCTCTCTGTACCAGCTGGAAAACTACTGCAACGAATTCGGATCTGGT
11	pRDV_f_NcoI	AGAAGGAGATATATCCATGG
12	pRDV_r_EcoRI	TGGCCACCAGATCCGAATTC

Restriction sites are underlined.

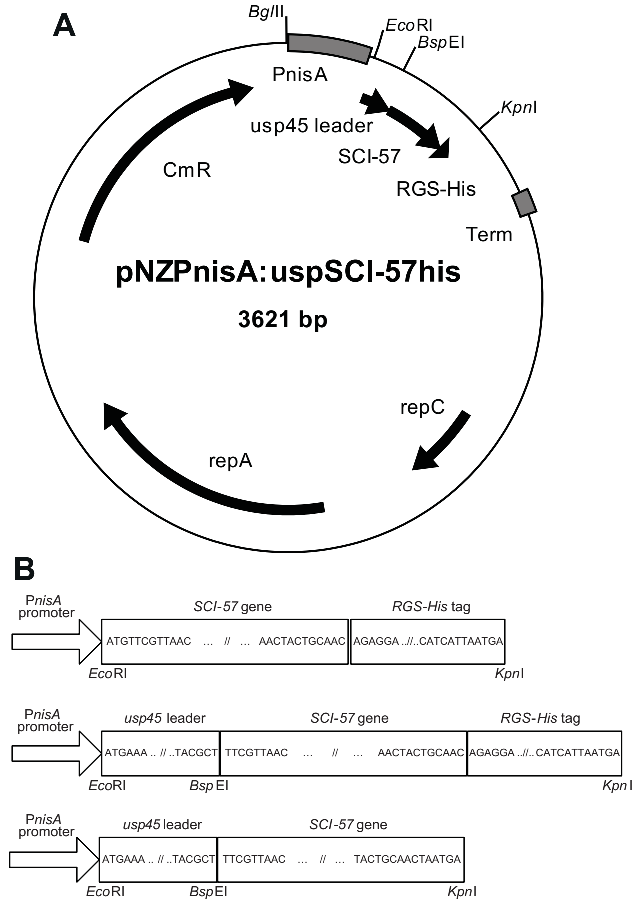

Figure 1. Schematic representation of the various constructs used in this study. **A**: Representative map of pNZPnisA:uspSCI-57his showing promoter P*nisA*, *usp45* leader signal, *SCI-57* gene, and *RGS-His* tag in the modified pNZ8048 backbone. **B**: Partial DNA sequences of the gene constructs inserted into the modified pNZ8048 backbone. From top to bottom: pNZPnisA:SCI-57his, pNZPnisA:uspSCI-57his, and pNZPnisA:uspSCI-57. For pNZPnisA:SCI-57his and pNZPnisA:uspSCI-57his, gene constructs were generated by assembly PCR, digested with *Kpn*I and *Bgl*II, and ligated into the similarly digested pNZPnisA:CYTO-LLO vector. For pNZPnisA:uspSCI-57, the assembly PCR product was digested by *Kpn*I and *Bsp*EI and ligated into a similarly digested pNZPnisA:uspSCI-57his vector. All plasmids were maintained in *E. coli* EC1000 and subsequently transformed into electrocompetent *L. lactis* NZ9000.

Growth Curve Determination

Overnight cultures of *L. lactis* NZ9000(pNZPnisA:uspSCI-57his) were diluted 1:25 into fresh GM17Cm medium. Nisin (Sigma, St. Louis, MO) at various concentrations was added at indicated times. Cultures were grown statically at 30°C up to 4 h after the latest induction point. Growth curves were determined by taking measurements of the optical density at 600 nm (OD_{600}) on a plate reader (Infinite M200, Tecan, Männedorf, Switzerland). The growth curves were fitted in Matlab (MathWorks, Natick, MA) using a logistic equation, $K/(1+e^{-r(t-l)})$, and the doubling times were calculated using $\ln(2)/r$.

Detection of Secreted SCI-57

Overnight cultures of *L. lactis* NZ9000(pNZPnisA:uspSCI-57his or pNZPnisA:SCI-57his) were diluted 1:25 into fresh GM17Cm medium. For buffering with sodium phosphates, 1 M NaH_2PO_4 and 1 M Na_2HPO_4 were mixed at a molar ratio of 1:19 and added at a final concentration of 50 mM to achieve the desired pH. Cultures were grown to mid-log phase ($OD_{600} \approx 0.4$–0.5) for 2.5 h and induced with 1 or 10 ng/mL nisin for 4 more hours. When investigating the effect of pH modulation, 2% or 10% culture volume of 5 N NaOH was added. At specified time points, aliquots were taken and OD_{600} and pH were measured. Cells were removed from the supernatant by a 10-min centrifugation at 4°C and 5,000*g*. The supernatant was then passed through a 0.22-μm filter (Millipore, Billerica, MA) to remove any cells, and 15.6 μL supernatant was mixed with reducing agent and lithium dodecyl sulfate (LDS) sample buffer (as recommended by manufacturer) for analysis by SDS–PAGE in a 12% NuPAGE® Bis-Tris gel (Invitrogen, Carlsbad, CA). Proteins were then transferred to a nitrocellulose membrane (Invitrogen). RGS-His-tagged proteins were analyzed by Western blotting with the RGS-His antibody (Qiagen, #34610) and then IRDye800-conjugated goat-anti-mouse immunoglobulin G secondary antibody (Rockland, Gilbertsville, PA, #610–131–121). The blot was then scanned on an Odyssey infrared imager (LI-COR Biosciences, Lincoln, NE) and the proteins were quantified by their relative intensities on the IR800 channel.

Cell Culture

The murine 3T3-L1 preadipocyte cell line (courtesy of Dr. Christopher Chen, University of Pennsylvania) was maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% calf serum (HyClone, Logan, UT), penicillin (100 U/mL), and streptomycin (100 μg/mL), with a change of medium every 3 days. The cells were differentiated using a slight modification of a published method (Nakashima et al., 2000). Briefly, 3T3-L1 preadipocytes were allowed to grow for 2 days post-confluency and were then differentiated by addition of the same medium containing isobutylmethylxanthine (500 μM), dexamethasone (1 μM), and insulin (1 μg/mL) for 2 days and then medium containing only insulin additive for 3 additional days. The medium was then changed every 3 days until the cells contained large oil locules characteristic of fully differentiated adipocytes, typically around 9–12 days.

Bioactivity of Secreted SCI-57 on 3T3-L1 Cells

3T3-L1 preadipocytes were seeded on a six-well plate and differentiated as described above. Fully differentiated 3T3-L1 adipocytes were serum starved overnight with 0.5% calf serum in DMEM. Supernatant of *L. lactis*

NZ9000(pNZPnisA:uspSCI-57his or pNZPnisA:uspSCI-57) was prepared as described above. The supernatant was then concentrated 20-fold in a 3-kDa cutoff filter (Millipore) and 100 μL was added along with 2 mL DMEM to the serum-starved 3T3-L1 cells. Dilution into DMEM also ensured that all signaling assays were performed at near-neutral pH. After a 15-min incubation at 37°C, cells were washed once in PBS and lysed in cell extraction buffer (Invitrogen) supplemented with a protease inhibitor cocktail (Sigma, #P8340), phosphatase inhibitor cocktails (Sigma, #P0044 and #P5726), and phenylmethanesulfonylfluoride (Amresco, Solon, OH). Cell debris was removed by centrifugation at 14,000*g* and 4°C for 15 min. Cell lysate (20–50 μg, but constant for a given experiment) was mixed with reducing agent and LDS sample buffer for analysis by SDS–PAGE in a 4–12% Bis-Tris gel (Invitrogen). Proteins were then transferred to a nitrocellulose membrane. Phosphorylated Akt (p-Akt) was quantified by Western blotting, as described above, using p-Akt(Ser473) or total Akt primary antibodies (Cell Signaling Technology, Danvers, MA, #4051 and #9272, respectively) and then IRDye800-conjugated goat-anti-mouse (same as above) or goat-anti-rabbit immunoglobulin G secondary antibody (Rockland, #611–132–122). The proteins were quantified by their relative intensities on the IR800 channel.

Results

Effect of Nisin Addition on *L. lactis* Growth Rate

The growth rate of *L. lactis* NZ9000(pNZPnisA:uspSCI-57his) as a function of inducer concentration and time of induction was monitored by taking OD_{600} measurements every 30 min for 7 h. When added at 1 h, nisin affected cell growth in a dose-dependent manner, with the largest inhibitory effect occurring at the highest inducer concentration (10 ng/mL, Fig. S1A). Lower OD_{600} readings were observed as early as the first time point after nisin addition (within 30 min) and this attenuated signal persisted until the last time point (7 h), indicating that nisin acted immediately and continuously on the culture until saturation. However, when the culture was induced at 2.5 h, the extent to which nisin adversely affected growth rate and saturated culture density was noticeably mitigated (Fig. S1B). We therefore tested a wider range of inducer concentrations (0.001, 0.01, 0.1, 0.5, 1, 5, or 10 ng/mL nisin) and induction start times (0.5, 1, 1.5, 2, 2.5, or 3 h) and we quantified the doubling time for each combination of these two variables (Fig. 2). Low nisin concentrations ($\leq$0.1 ng/mL) had a minimal effect on cell growth at all induction times. High nisin concentrations ($\geq$ 0.5 ng/mL) resulted in significantly longer doubling times at early induction start times, but there was a clear inverse correlation between induction start time and culture doubling time.

Secretion of SCI-57 by *L. lactis* into Supernatant

L. lactis NZ9000(pNZPnisA:uspSCI-57his or pNZPnisA:SCI-57his) (Fig. 1B) were grown and induced with 0, 1, or 10 ng/mL nisin at 2.5 h as described above. The 2.5-h induction start time was initially chosen because it seemed to balance growth rate (cultures are minimally affected even at 10 ng/mL nisin) and total induction time (SCI-57 expression can still proceed for several hours in a

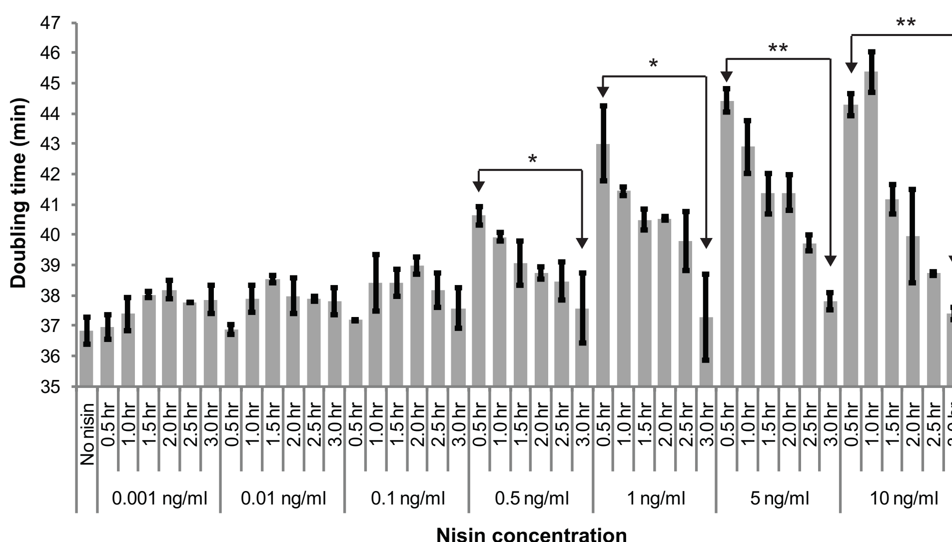

Figure 2. Effect of nisin concentration and induction time on growth rate. Various nisin concentrations (0–10 ng/mL) were added at different times after 1:25 dilution of an overnight culture of NZ9000(pNZPnisA:uspSCI-57his). Cultures were grown statically at 30°C for a total of 7 h. Growth was determined by measuring the optical density at 600 nm over time. The growth curves were fitted to a logistic equation $K/(1+e^{-r(t-t_0)})$, where r is the growth rate, and the doubling times were calculated from ln(2)/r. $^{*}P<0.05$ and $^{**}P<0.01$ for statistical comparison of doubling times after 0.5 and 3 h induction at each nisin concentration using a one-tailed Student's *t*-test.

log-phase culture, Fig. S1B and Fig. 2). After induction for 4 additional hours, cells were removed by centrifugation and secreted SCI-57 was detected via the C-terminal RGS-His tag using Western blotting. Bands of ≈7–8 kDa (expected: 7.5 kDa) were detected in cultures induced with 1 or 10 ng/mL nisin, while no signal was detected in the nisin-free control (Fig. 3). This confirms that there is no detectable leaky expression from the P*nisA* promoter and that nisin is necessary for inducing SCI-57 expression. In addition, no signal was detected in the supernatant from *L. lactis* harboring pNZPnisA:SCI-57his (without *usp45* secretion leader), whether or not nisin was added, indicating that the usp45 signal peptide is necessary for secretion of the downstream protein, which is in agreement with previous studies (Le Loir et al., 2001; van Asseldonk et al., 1993). The molecular weight of RGS-His-tagged SCI-57 with the usp45 signal peptide is 10.3 kDa. All of our observed bands are below the 10 kDa mark, indicating that the usp45 signal peptide has been cleaved, as expected, from SCI-57 before or during secretion into the supernatant (Fig. 3). Since acidification of the medium by *L. lactis* may impact cell growth and/or protein secretion, we also tested the effect of adding sodium hydroxide or sodium phosphates to GM17 media. There was no improvement in growth rate and a slight decrease in secreted SCI-57 (Fig. 3).

Bioactivity of Secreted SCI-57 on Adipocytes

To investigate if secreted SCI-57 is properly folded and biologically active, its ability to functionally signal was tested on differentiated 3T3-L1 adipocytes. Insulin signals by binding and activating cell-surface insulin receptors, which phosphorylate adapter proteins such as the insulin receptor substrate (IRS) family, which then recruit and activate downstream effector molecules. One such effector protein that is required for insulin signaling is phosphatidylinositol 3-kinase (PI 3-kinase), which phosphorylates Akt (Jiang et al., 2003; Summers et al., 1998). In our assay, we added conditioned *L. lactis* medium to differentiated 3T3-L1 adipocytes and used p-Akt as a metric of insulin signaling.

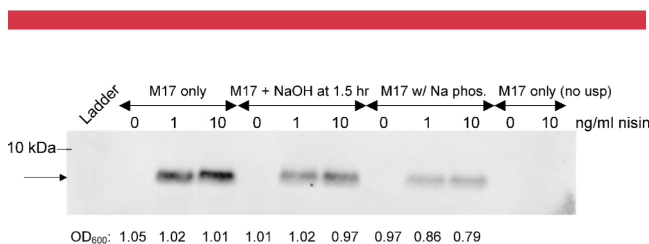

Figure 3. Western blot detection of SCI-57 in supernatant. Overnight cultures of NZ9000(pNZPnisA:SCI-57his or pNZPnisA:uspSCI-57his) were diluted 1:25, grown in GM17Cm media with or without 50 mM sodium phosphates for 2.5 h, and then induced with 1 or 10 ng/mL nisin for an additional 4 h. For modulating pH with NaOH, 10% culture volume of 5 N NaOH was added at 1.5 h after induction. After centrifugation, the supernatant was filtered and used for Western blotting. Anti-RGS-His antibody was used to detect SCI-57 secreted into the supernatant. The molecular weight of RGS-His-tagged SCI-57, after cleavage of usp45 signal peptide, is 7.5 kDa. Arrow shows ≈7–8 kDa.

Serum-starved, fully differentiated 3T3-L1 adipocytes were incubated with 1 nM commercial insulin solution or 100 μL 20-fold-concentrated supernatant for 15 min at 37°C and p-Akt was detected by Western blotting of cell lysates. Both commercial insulin and supernatant from induced NZ9000(pNZPnisA:uspSCI-57his) treated with NaOH showed a strong p-Akt signal (Fig. 4A,B). Importantly, no signals could be seen in supernatants from uninduced NZ9000(pNZPnisA:uspSCI-57his) treated with NaOH or from induced cells without buffering or with sodium phosphate buffering, even though these latter cultures clearly secrete the full-length polypeptide (Fig. 3). These results indicate that functional folding of secreted SCI-57 is an important bottleneck in obtaining bioactive product from *L. lactis*, but this bottleneck is dependent on the pH and buffer conditions in the medium. To estimate the concentration of active RGS-His-tagged SCI-57 in the supernatant, we quantified p-Akt signals from the Western blots of three independent experiments and found the signal of SCI-57 to be roughly equivalent to 1 nM commercial insulin (Fig. 4B). Since 100 μL of 20-fold-concentrated supernatant was added to 2 mL DMEM, the original concentration of functional SCI-57 secreted by

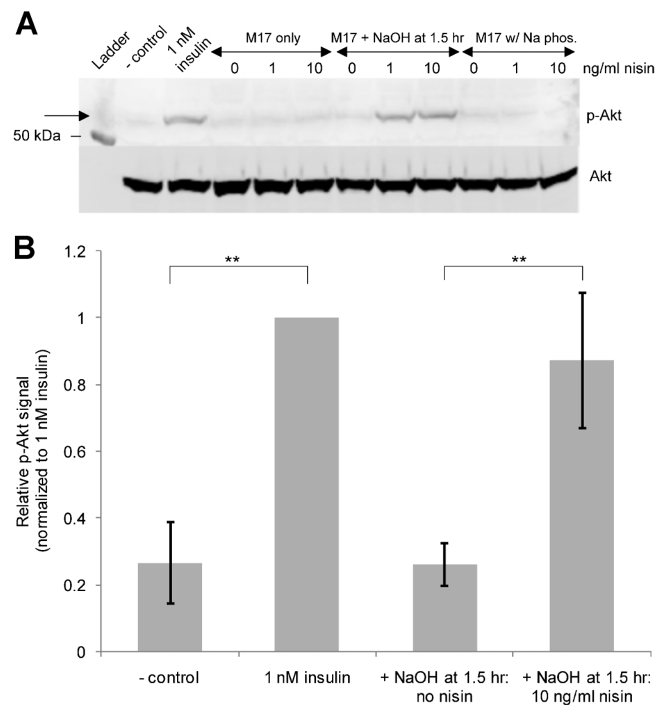

Figure 4. In vitro biological activity of secreted SCI-57. **A**: Representative blot of p-Akt signaling. Supernatant of NZ9000(pNZPnisA:uspSCI-57his or pNZPnisA:uspSCI-57) cultures were prepared as described in Fig. 3, and were then concentrated 20-fold with a 3-kDa cutoff filter. Concentrated supernatant (100 μL) was added to 2 mL DMEM on fully differentiated, serum-starved 3T3-L1 adipocytes. After a 15-min incubation at 37°C, cells were lysed and the lysates were blotted for p-Akt(Ser473). **B**: Quantification of p-Akt signaling from three independent experiments. Relative intensities are shown by normalizing to the positive control (1 nM commercial insulin). $^{**}P < 0.01$.

NZ9000(pNZPnisA:uspSCI-57his) was approximately 1 nM.

Effect of pH Modulation on Functional SCI-57 Expression

The previous results (Figs. 3 and 4) indicate that the biological activity of SCI-57 is more heavily affected by the medium pH and buffering conditions than by the overall protein secretion level. We therefore investigated the biological activity of various conditioned *L. lactis* media, each subject to a different pH profile, on 3T3-L1 adipocytes. Two modes of pH modulation were tested: (1) continuous control, in which 2% culture volume of 5 N NaOH was added hourly for 5 h; or (2) a single pulse, in which 10% culture volume of 5 N NaOH was added once at the indicated time after induction. In effect, the same total amount of NaOH was added in each case. As seen from the OD_{600} readings (Fig. 5A), early addition of NaOH (continuous or single dose at 0.5 h) has an inhibitory effect on growth. Corresponding to the lower OD_{600} readings, the amount of secreted SCI-57 detected for culture with early NaOH addition was also lower on a per-volume basis. However, when the biological activity of these samples was tested using our p-Akt signaling assay on 3T3-L1 adipocytes (Fig. 5B), the observed trend was the opposite of that seen in Fig. 5A. Cultures grown with early NaOH addition secrete similar, if not higher, levels of functional SCI-57 than cultures grown with late NaOH addition. This further confirms that the biological activity of secreted SCI-57 is more heavily affected by buffering conditions than total secretion level and suggests that earlier counterbalancing of the natural medium acidification increases the fraction of functional SCI-57 molecules.

Further Enhancement of SCI-57 Bioactivity by Removal of RGS-His Tag

Finally, we investigated whether the addition of the RGS-His tag, which is convenient for detection and assay development but not desirable for eventual in vivo applications, has any effect on the bioactivity of SCI-57. The p-Akt signaling experiments on 3T3-L1 adipocytes were therefore repeated with conditioned media from NZ9000(pNZPnisA:uspSCI-57) cultures. Interestingly, not only was p-Akt signaling much stronger for cultures treated with NaOH but it was now also detectable using untreated cultures or cultures with phosphate buffer (Fig. 6). This suggests that the RGS-His tag adversely affects insulin receptor binding and/or SCI-57 folding.

Discussion

As shown in Fig. 2, both nisin concentration and time of induction affect the growth rate. For a given induction time, a higher nisin concentration increases the doubling time (i.e., slows the growth rate). This is possibly due to a combination of increased burden from producing heterologous protein and nisin toxicity [nisin can inhibit bacterial cell wall biosynthesis and can kill cells by forming pores in the cytoplasmic membrane (McAuliffe et al., 2001)]. For a given nisin concentration, a later induction time reduces the doubling time (i.e., increases the growth rate). There is perhaps a critical OD_{600} threshold in early- to mid-log phase above which the burdens imposed by heterologous protein synthesis and nisin toxicity are offset by the faster growth rate in this phase.

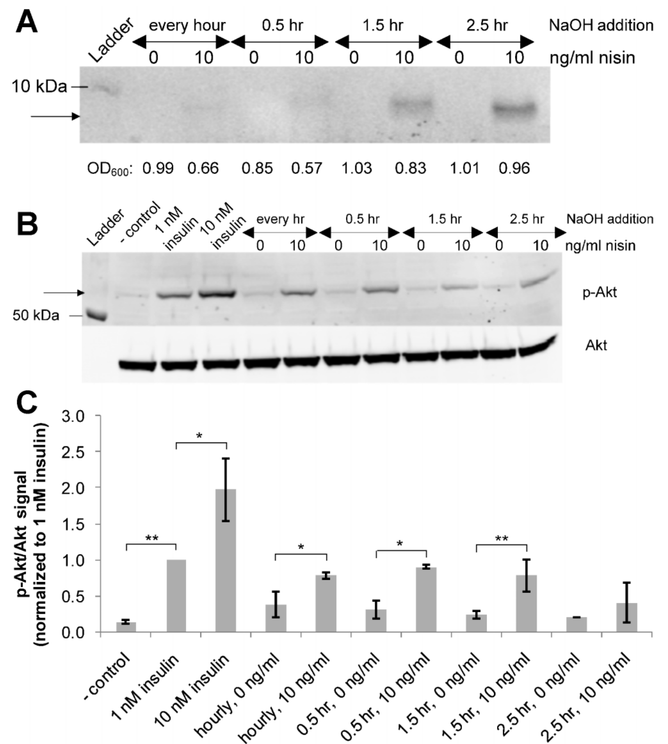

Figure 5. Effect of temporal pH modulation on SCI-57 secretion and bioactivity. **A**: Western blot showing secreted SCI-57 with different NaOH treatments, with final OD_{600} readings given below. Overnight cultures of NZ9000(pNZPnisA:uspSCI-57his) were diluted 1:25 and grown in GM17Cm media for 2.5 h and induced with 0 or 10 ng/mL nisin for an additional 4 h. For continuous pH regulation with NaOH, 2% culture volume of 5 N NaOH was added at 1.5, 2.5, 3.5, 4.5, and 5.5 h after the 1:25 dilution. For single-dose modulation with NaOH, 10% culture volume of 5 N NaOH was added at 0.5, 1.5, or 2.5 h after nisin induction. After centrifugation, the supernatant was filtered and used directly for Western blotting. RGS-His antibody was used to detect the RGS-His-tagged SCI-57 secreted into the supernatant. **B**: Representative Western blot showing relative p-Akt signals on differentiated 3T3-L1 adipocytes stimulated by supernatant in different buffering conditions. Supernatant of NZ9000(pNZPnisA:uspSCI-57his) culture was prepared as described in Fig. 5A, concentrated 20-fold, and 100 µL was added along with 2 mL DMEM to 3T3-L1 cells. After a 15-min incubation at 37°C, cells were lysed and the lysate subjected to Western blotting and detection with a p-Akt antibody. **C**: Quantification of p-Akt signals, as shown in (B), from two independent experiments. Notation for the last eight samples is: NaOH addition time after nisin induction, nisin concentration. $^{*}P < 0.05$ and $^{**}P < 0.01$.

The correlation between pH regulation and SCI-57 bioactivity is summarized in Table III. *L. lactis* naturally acidifies the GM17 medium to pH <5.7 when the culture is saturated. Regulating the pH with a base or buffering the media allows *L. lactis* to grow to a much higher density

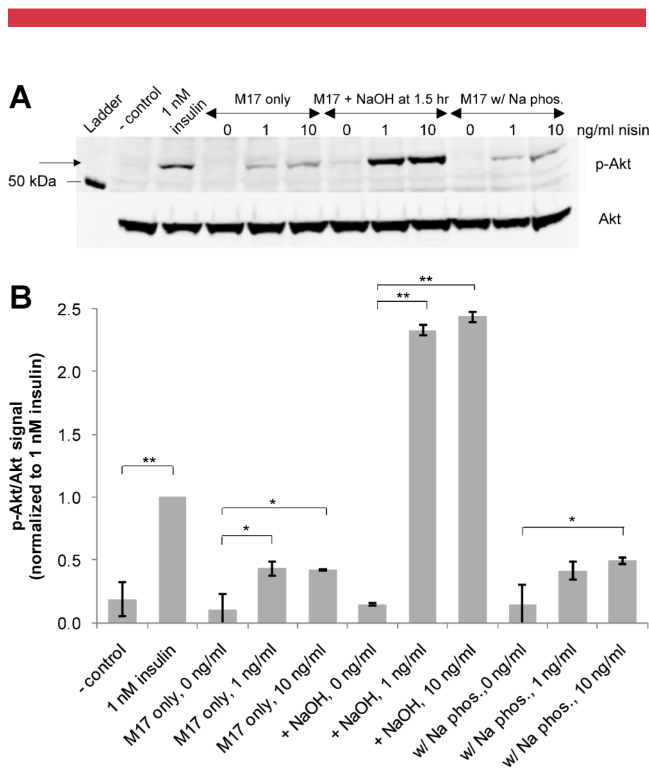

Figure 6. Biological activity of secreted SCI-57 without the RGS-His tag. **A**: Representative Western blot showing relative p-Akt signals on differentiated 3T3-L1 adipocytes stimulated by supernatant from NZ9000 [pNZPnisA:uspSCI-57 (no RGS-His tag)] cultures. Supernatants were prepared as described in Fig. 3 and signaling experiments were carried out as described in Fig. 4A. **B**: Quantification of p-Akt signals, as shown in (A), from two independent experiments. Notation for the last nine samples is: medium composition, nisin concentration. $^{*}P < 0.05$ and $^{**}P < 0.01$.

(Tremillon et al., 2010) and it has also been shown to increase the stability and biological activity of secreted interleukin-10 (Schotte et al., 2000; Steidler et al., 2000). In our study, buffering the medium may increase the solubility, stability, and/or folding of secreted SCI-57. SCI-57 has a pI similar to native insulin and is most soluble at neutral pH (Hua et al., 2008). Also, wild-type insulin is most stable near neutral pH, due to deamidation at low pH and aggregation at high pH (Brange and Langkjaer, 1992). However, since the pH during our induction experiments stays relatively close to neutral pH, deamidation and aggregation are unlikely to have large effects. Furthermore, SCI-57 was designed with amino acid substitutions in the A and B chains to prevent dimerization and higher order assembly (Hua et al., 2008). Thus, we hypothesize that the marked increase in biological activity upon addition of NaOH is primarily due to better folding. SCI-57, like native insulin, requires three disulfide bonds (A6–A11, A7–B7, A20–B19) for correct folding and biological activity. Previous work indicated that basic pH facilitates folding of insulin by deprotonating thiolate moieties and thus limiting aggregation of reduced B chains, giving rise to more free B chains that can form productive disulfide bonds with A chains (Hua et al., 2002; Weiss, 2009). Also, thiol-disulfide exchange, the principal mechanism by which disulfide bonds are formed and rearranged in proteins, proceeds via a nucleophilic attack of the thiolate anion and alkaline conditions facilitate this reaction (Rudolph and Lilie, 1996). In our case, the addition of NaOH could provide a transient alkaline environment to more efficiently facilitate formation of the disulfide bonds of SCI-57, thus giving rise to higher biological activity. During the folding of insulin, there exists a critical folding intermediate containing the single disulfide A20–B19, which has been suggested to form first and guide subsequent folding (Yan et al., 2003). The posited initial formation of this disulfide bond may also explain why SCI-57 without the RGS-His tag exhibits higher biological activity even without pH modulation. The RGS-His tag in our experiments is fused directly to the C-terminus of SCI-57 (i.e., after A21), which might impede the formation of this initial disulfide bond.

Our studies demonstrate proof of principle that food-grade microorganisms can be engineered to secrete bioactive insulin analogs, and we are carrying out further in vitro optimization and in vivo testing of these constructs. One notable difference between the in vitro experiments presented here and the actual in vivo environment is the pH-sensitive bioactivity. While pH decreases in our current experiments significantly hindered the bioactivity (e.g., without intervention with NaOH), the gut is highly pH-regulated and will not be affected by the acidification that occurs in unbuffered M17 medium in vitro. Furthermore, *L. lactis* resides in the jejunum and ileum of human intestine (Wells and Mercenier, 2008), which have pH values of ~6.2 and ~6.8–8.4, respectively (Charman et al., 1997). These pH values were closely approximated in the experiments in which we observed functional SCI-57. In addition, SCI-57 administered in vivo will not have an RGS-His tag,

Table III. pH values during different induction conditions.

	Starting pH	Ending pH	pH spike	Insulin activity (with RGS-His tag)	Insulin activity (no tag)
M17 only	6.9	5.7	No	−	+
M17 with 50 mM sodium phosphates	7.2	6.5	No	−	+
M17 with NaOH addition every hour	6.9	7–7.7	No	++	N.D.
M17 with NaOH addition at 0.5 h	6.9	7–9.17	>9	++	N.D.
M17 with NaOH addition at 1.5 h	6.9	6.9–7.1	>7.7	++	+++
M17 with NaOH addition at 2.5 h	6.9	7.2–7.7	>7.7	+	N.D.

−: no activity; +: equivalent to <1 nM commercial insulin; ++: equivalent to ≈1 nM commercial insulin; +++: equivalent to >1 nM commercial insulin; N.D.: not determined.

elimination of which should substantially increase the biological activity of the protein. Additionally, to avoid potential complications with induction in vivo, it may be possible to preinduce *L. lactis* with nisin prior to oral administration. For example, it has been previously shown that treating *L. lactis* with a 1-h pulse of nisin can induce protein secretion for 10 h (Bermudez-Humaran et al., 2003).

In summary, we have constructed a *L. lactis* strain that can efficiently secrete SCI-57 that is biologically active at the physiological pH in the gut. From a biomedical perspective, the advantages of this system for oral insulin delivery are threefold. First, *L. lactis* has long been shown to have a safe association with humans, and thus possible adverse effects (e.g., using synthetic particles) can be minimized. Second, the protein can by secreted locally in the small intestine, minimizing loss during passage through the upper digestive tract and potentially enabling 'on demand' secretion. Third, as a live delivery vehicle, the pharmacokinetics can be genetically tuned to match delivery requirements. From a biotechnological perspective, using SCI-57-secreting *L. lactis* eliminates the needs for two-chain synthesis, expensive protein purification, and temperature-sensitive storage of insulin, offering a cheaper and more convenient alternative to traditional insulin replacement therapy.

We are grateful to Dr. Cormac Gahan for providing the plasmid pNZPnisA:CYTO-LLO, Dr. Jan Kok for providing the strain EC1000, and Dr. Christopher Chen for supplying the 3T3-L1 cell line. This work was supported by a grant from the American Diabetes Association (#7–09-IN-24), a National Science Foundation CAREER Award (#1055231), and startup funds from the University of Pennsylvania to C.A.S.

References

Bahey-El-Din M, Griffin BT, Gahan CG. 2008. Nisin inducible production of listeriolysin O in *Lactococcus lactis* NZ9000. Microb Cell Fact 7:24.

Balcazar JL, de Blas I, Ruiz-Zarzuela I, Vendrell D, Calvo AC, Marquez I, Girones O, Muzquiz JL. 2007. Changes in intestinal microbiota and humoral immune response following probiotic administration in brown trout (*Salmo trutta*). Br J Nutr 97(3):522–557.

Bermudez-Humaran LG, Langella P, Commissaire J, Gilbert S, Le Loir Y, L'Haridon R, Corthier G. 2003. Controlled intra- or extracellular production of staphylococcal nuclease and ovine omega interferon in *Lactococcus lactis*. FEMS Microbiol Lett 224(2):307–313.

Binz HK, Amstutz P, Kohl A, Stumpp MT, Briand C, Forrer P, Grutter MG, Plückthun A. 2004. High-affinity binders selected from designed ankyrin repeat protein libraries. Nat Biotechnol 22(5):575–582.

Braat H, Rottiers P, Hommes DW, Huyghebaert N, Remaut E, Remon JP, van Deventer SJ, Neirynck S, Peppelenbosch MP, Steidler L. 2006. A phase I trial with transgenic bacteria expressing interleukin-10 in Crohn's disease. Clin Gastroenterol Hepatol 4(6):754–759.

Brange J, Langkjaer L. 1992. Chemical stability of insulin. 3. Influence of excipients, formulation, and pH. Acta Pharm Nord 4(3):149–158.

Charman WN, Porter CJ, Mithani S, Dressman JB. 1997. Physiochemical and physiological mechanisms for the effects of food on drug absorption: The role of lipids and pH. J Pharm Sci 86(3):269–282.

de Ruyter PG, Kuipers OP, Beerthuyzen MM, van Alen-Boerrigter I, de Vos WM. 1996a. Functional analysis of promoters in the nisin gene cluster of *Lactococcus lactis*. J Bacteriol 178(12):3434–3439.

de Ruyter PG, Kuipers OP, de Vos WM. 1996b. Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. Appl Environ Microbiol 62(10):3662–3667.

Hua Q. 2010. Insulin: A small protein with a long journey. Protein Cell 1(6):537–551.

Hua QX, Chu YC, Jia W, Phillips NF, Wang RY, Katsoyannis PG, Weiss MA. 2002. Mechanism of insulin chain combination. Asymmetric roles of A-chain alpha-helices in disulfide pairing. J Biol Chem 277(45):43443–43453.

Hua QX, Nakagawa SH, Jia W, Huang K, Phillips NB, Hu SQ, Weiss MA. 2008. Design of an active ultrastable single-chain insulin analog: Synthesis, structure, and therapeutic implications. J Biol Chem 283(21):14703–14716.

Jiang ZY, Zhou QL, Coleman KA, Chouinard M, Boese Q, Czech MP. 2003. Insulin signaling through Akt/protein kinase B analyzed by small interfering RNA-mediated gene silencing. Proc Natl Acad Sci USA 100(13):7569–7574.

Khafagy el S, Morishita M, Onuki Y, Takayama K. 2007. Current challenges in non-invasive insulin delivery systems: A comparative review. Adv Drug Deliv Rev 59(15):1521–1546.

Klijn N, Weerkamp AH, de Vos WM. 1995. Genetic marking of *Lactococcus lactis* shows its survival in the human gastrointestinal tract. Appl Environ Microbiol 61(7):2771–2774.

Kuipers OP, de Ruyter PG, Kleerebezem M, de Vos WM. 1998. Quorum sensing-controlled gene expression in lactic acid bacteria. J Biotechnol 64:15–21.

Le Loir Y, Nouaille S, Commissaire J, Bretigny L, Gruss A, Langella P. 2001. Signal peptide and propeptide optimization for heterologous protein secretion in *Lactococcus lactis*. Appl Environ Microbiol 67(9):4119–4127.

Leenhouts K, Buist G, Bolhuis A, ten Berge A, Kiel J, Mierau I, Dabrowska M, Venema G, Kok J. 1996. A general system for generating unlabelled gene replacements in bacterial chromosomes. Mol Gen Genet 253(1–2):217–224.

McAuliffe O, Ross RP, Hill C. 2001. Lantibiotics: Structure, biosynthesis and mode of action. FEMS Microbiol Rev 25(3):285–308.

Mierau I, Kleerebezem M. 2005. 10 years of the nisin-controlled gene expression system (NICE) in *Lactococcus lactis*. Appl Microbiol Biotechnol 68(6):705–717.

Morishita M, Peppas NA. 2006. Is the oral route possible for peptide and protein drug delivery? Drug Discov Today 11(19–20):905–910.

Muller G. 2010. Oral delivery of protein drugs: Driver for personalized medicine. Curr Issues Mol Biol 13(1):13–24.

Nakashima N, Sharma PM, Imamura T, Bookstein R, Olefsky JM. 2000. The tumor suppressor PTEN negatively regulates insulin signaling in 3T3-L1 adipocytes. J Biol Chem275(17):12889–12895.

Nouaille S, Ribeiro LA, Miyoshi A, Pontes D, Le Loir Y, Oliveira SC, Langella P, Azevedo V. 2003. Heterologous protein production and delivery systems for *Lactococcus lactis*. Genet Mol Res 2(1):102–111.

Pope B, Kent HM. 1996. High efficiency 5 min transformation of *Escherichia coli*. Nucleic Acids Res 24(3):536–537.

Rajpal G, Liu M, Zhang Y, Arvan P. 2009. Single-chain insulins as receptor agonists. Mol Endocrinol 23(5):679–688.

Rudolph R, Lilie H. 1996. In vitro folding of inclusion body proteins. FASEB J 10(1):49–56.

Schotte L, Steidler L, Vandekerckhove J, Remaut E. 2000. Secretion of biologically active murine interleukin-10 by *Lactococcus lactis*. Enzyme Microb Technol 27(10):761–765.

Steidler L, Hans W, Schotte L, Neirynck S, Obermeier F, Falk W, Fiers W, Remaut E. 2000. Treatment of murine colitis by *Lactococcus lactis* secreting interleukin-10. Science 289(5483):1352–1355.

Steidler L, Neirynck S, Huyghebaert N, Snoeck V, Vermeire A, Goddeeris B, Cox E, Remon JP, Remaut E. 2003. Biological containment of genetically modified *Lactococcus lactis* for intestinal delivery of human interleukin 10. Nat Biotechnol 21(7):785–789.

Still JG. 2002. Development of oral insulin: Progress and current status. Diabetes Metab Res Rev 18 (Suppl. 1): S29–S37.

Summers SA, Garza LA, Zhou H, Birnbaum MJ. 1998. Regulation of insulin-stimulated glucose transporter GLUT4 translocation and Akt kinase activity by ceramide. Mol Cell Biol 18(9):5457–5464.

Tremillon N, Issaly N, Mozo J, Duvignau T, Ginisty H, Devic E, Poquet I. 2010. Production and purification of staphylococcal nuclease in *Lactococcus lactis* using a new expression-secretion system and a pH-regulated mini-reactor. Microb Cell Fact 9:37.

van Asseldonk M, Rutten G, Oteman M, Siezen RJ, de Vos WM, Simons G. 1990. Cloning of usp45, a gene encoding a secreted protein from *Lactococcus lactis* subsp. lactis MG1363. Gene 95(1):155–160.

van Asseldonk M, de Vos WM, Simons G. 1993. Functional analysis of the *Lactococcus lactis* usp45 secretion signal in the secretion of a homologous proteinase and a heterologous alpha-amylase. Mol Gen Genet 240(3):428–434.

Weiss MA. 2009. Proinsulin and the genetics of diabetes mellitus. J Biol Chem 284(29):19159–19163.

Wells JM, Mercenier A. 2008. Mucosal delivery of therapeutic and prophylactic molecules using lactic acid bacteria. Nat Rev Microbiol 6(5):349–362.

Yan H, Guo ZY, Gong XW, Xi D, Feng YM. 2003. A peptide model of insulin folding intermediate with one disulfide. Protein Sci 12(4):768–775.