β-Carotene–Producing Bacteria Residing in the Intestine Provide Vitamin A to Mouse Tissues In Vivo1–3

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Abstract

Vitamin A deficiency (VAD) is an overwhelming public health problem that affects hundreds of millions of people worldwide. A definitive solution to VAD has yet to be identified. Because it is an essential nutrient, vitamin A or its carotenoid precursor β-carotene can only be obtained from food or supplements. In this study, we wanted to establish whether β-carotene produced in the mouse intestine by bacteria synthesizing the provitamin A carotenoid could be delivered to various tissues within the body. To achieve this, we took advantage of the Escherichia coli MG1655*, an intestine-adapted spontaneous mutant of E. coli MG1655, and the plasmid pAC-BETA, containing the genes coding for the 4 key enzymes of the β-carotene biosynthetic pathway (geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, and lycopene cyclase) from Erwinia herbicola. We engineered the E. coli MG1655* to produce β-carotene during transformation with pAC-BETA (MG1655*–βC) and gavaged wild-type and knockout mice for the enzyme β-carotene 15,15 ‘-oxygenase with this recombinant strain. Various regimens of bacteria administration were tested (single vs. multiple and low vs. high doses). β-Carotene concentration was measured by HPLC in mouse serum, liver, intestine, and feces. Enumeration of MG1655*–βC cells in the feces was performed to assess efficiency of intestinal colonization. We demonstrated in vivo that probiotic bacteria could be used to deliver vitamin A to the tissues of a mammalian host. These results have the potential to pave the road for future investigations aimed at identifying alternative, novel approaches to treat VAD. J. Nutr. 144: 608–613, 2014.

Introduction

Vitamin A is an essential nutrient that is required for a number of crucial biologic functions in mammals, including normal organogenesis, immune competence, tissue differentiation, and the visual cycle (1, 2). Vitamin A and its derivatives (retinoids) are obtained from the diet as either preformed vitamin A (retinyl esters, retinol, and very small amounts of retinoic acid) from animal products or provitamin A carotenoids from vegetables and fruits (3, 4). β-Carotene is the most abundant vitamin A carotenoid precursor in the human diet (3). Within the enterocytes of the intestinal mucosa, β-carotene can be converted to retinoids by the action of the enzyme β-carotene 15,15 ‘-oxygenase (CMOI).10 This enzyme cleaves the provitamin A symmetrically, generating 2 molecules of retinaldehyde (3, 5, 6). During intestinal absorption, both preformed vitamin A and provitamin A carotenoids are ultimately converted into retinyl esters within the enterocytes. Here, these are incorporated into chylomicrons, lipoprotein particles that are secreted into the lymphatic system and then the bloodstream, to distribute vitamin A throughout the body (7).

Vitamin A deficiency (VAD) is a devastating public health problem that affects hundreds of millions of people and results in the loss of millions of lives every year (8). Current strategies for overcoming VAD include distribution of vitamin A or its precursor β-carotene via medicinal supplements, fortification of foods, and dietary diversification through horticulture and education programs (9, 10). These methods, which are successful if completed, are costly and difficult to extend into large-scale programs (9, 10). Therefore, they have not succeeded in eradicating VAD so far. Novel approaches to solve this problem are still needed.

In this study, we investigated whether β-carotene produced in the mouse intestine by bacteria synthesizing the provitamin A carotenoid could be delivered to various tissues within the body. We reasoned that, if in vivo approach such as this would be successful, it could lead the way for future investigations ultimately aimed at developing alternative strategies to treat VAD.
We took advantage of *Escherichia coli* MG1655*, an intestine-adapted spontaneous mutant of *E. coli* MG1655 (in turn derived from the K12 archetype) (11). We engineered *E. coli* MG1655* to produce β-carotene during transformation with pAC-BETA. This plasmid contains the genes coding for the 4 key enzymes of the β-carotene biosynthetic pathway [geranylgeranyl pyrophosphate synthase (*crtE*), phytoene synthase (*crtB*), phytoene desaturase (*crtI*), and lycopene cyclase (*crtY*)] from *Erwinia herbicola* (12). Previous studies showed that, when inserted into *E. coli* strains, this plasmid conferred to the bacteria the ability to synthesize β-carotene (12). The transformant strain (MG1655*-βC) synthesized the provitamin A carotenoid, as expected, and was administered by gavage to wild-type (WT) and CMOI knockout (CMOI/−) mice. β-Carotene concentration was measured by HPLC in mouse serum, liver, intestine, and feces. Efficiency of intestinal colonization was also estimated. We showed that β-carotene produced by these intestine-resident bacteria can be delivered to various tissues in mice. The strengths and limitations of this study are discussed.

**Materials and Methods**

**Bacterial strains, culture conditions, and vector.** An intestine-adapted spontaneous mutant of *E. coli* K12, strain MG1655* (11), was used as the host strain. This strain has the better ability to colonize the mouse intestine (11). Cultures were grown routinely at 37°C on Luria-Bertani (LB) medium (Life Technologies). The plasmid used was pAC-BETA, which contains the functional genes *crtE*, *crtB*, *crtI*, and *crtY* needed for β-carotene synthesis (12). Chemically competent MG1655* cells were transformed with pAC-BETA to yield the recombinant strain MG1655*-βC. These plasmid-transformed cells were grown at 37°C on LB containing 50 mg/L chloramphenicol (Cm) and 100 mg/L streptomycin sulfate (Str) for 3 d for maximum β-carotene production (antibiotics from Sigma). One hundred MG1655*-βC colonies were picked in duplicate onto LB medium supplemented with both 50 mg/L Cm and 100 mg/L Str and LB medium supplemented with 100 mg/L Str only for 3 generations to determine the stability of the pAC-BETA plasmid in the absence of selective pressure (Cm).

**Mice and diet.** WT and CMOI/− mice (5) were used in the current study. All mice were bred in our animal facility and had a mixed genetic background (C37BL/6 x sv129). Throughout the study, both water and diet were consumed ad libitum, and mice were maintained on a 12-h light/dark cycle from 0700 to 1900. All mice were maintained on a nonpurified diet copious in vitamin A (Prolab Isopro RMH3000 5p75; energy from protein, fat, and carbohydrates, 26, 14, and 60%, respectively; vitamin A, 9.98 μg/g diet; β-carotene, from trace to 2.6 μg/g diet) manufactured by LabDiet (W.F. Fisher and Son) (13). Experiments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (14) and were approved by the Rutgers University Institutional Committee on Animal Care.

**Administration of MG1655*-βC to WT mice.** Three 5-wk-old WT female mice were given drinking water containing 5 g/L Str (to eliminate resident facultative bacteria) and 1.25 g/L Cm (for stable maintenance of recombinant plasmid) for 24 h (day 1). After 18-h starvation of food and water, the mice were fed by oral gavage with 10^6 CFU of MG1655*-βC in 100 μL of PBS and 100 μL of 20% sucrose (day 3). This dose of the transformant bacteria was chosen based on the work of Fabich et al. (11), who performed experiments of mouse intestinal colonization with the parent bacterial strain MG1655*. After the gavage, both food and water (containing Str and Cm at the concentrations indicated above) were returned to the mice. Feces were collected on days 3, 4, 5, 6, 8, and 10, when mice were killed by CO₂ overdose to collect serum, liver, and (whole) intestinal mucosa (Fig. 1A). We labeled this regimen as low-dose 1 wk (LD-1).

**Administration of MG1655*-βC to mice lacking CMOI.** To overcome the high cleavage activity of CMOI in the mouse intestine (15) and, therefore, to increase the likelihood of detecting intact β-carotene in various tissues of the body, mice lacking CMOI were used. The MG1655*-βC transformants were administered to 3 5-wk-old CMOI/− female mice using the same experimental scheme outlined above (LD-1; Fig. 1A). Furthermore, to explore ways of increasing the amount of β-carotene detected in the tissues of the CMOI/− mice administered the β-carotene-producing bacteria, we followed 2 additional approaches. In the first approach, labeled high-dose (HD), the 5-wk-old CMOI/− female mice were administered a higher dose, i.e., 10^9 CFU of MG1655*-βC in 100μL of PBS and 100 μL of 20% sucrose following the same scheme described above. After gavage with the bacteria, these mice were divided into 2 groups. The first group was killed on day 10 [HD 1 wk (HD-1); n = 3], and the second group was killed on day 17 [HD 2 wk (HD-2); n = 3] of the experiment (Fig. 1A). In the second approach, labeled multidose (MD), the 5-wk-old CMOI/− female mice (n = 4) were administered the low dose (10^7 CFU) of the MG1655*-βC transformant strain multiple times, specifically on days 3, 6, and 9, and then killed on day 17 (Fig. 1B). Feces were collected on days 0, 4, 5, 7, 8, 10, 11, 12, 14, and 17 of the experiment (Fig. 1B).

**FIGURE 1** Outline of the experimental timeline of different regimens of MG1655*-βC administration. Single-dose regimens (A: LD-1, HD-1, and HD-2; MD regimen (B). From day 1 of each of the 4 different regimens, the mice were maintained on drinking water containing antibiotics (see Materials and Methods) throughout the duration of the experiment. Arrows indicate time of delivery of MG1655*-βC. Eighteen hours before the gavage, the mice were starved of food and water, which were returned to them after the oral administration of the bacteria. The day of feces collection is also indicated. Mice were killed on day 10 for the LD-1 and HD-1 regimens and on day 17 for the HD-2 and MD regimens. HD-1, high-dose 1 wk; HD-2, high-dose 2 wk; LD-1, low-dose 1 wk; MD, multi-dose.
Measurements of β-carotene production from MG1655*βC. MG1655*βC was propagated in 5 mL of LB broth supplemented with 50 mg/L Cm and 100 mg/L Str for 3 d at 37°C. MG1655*βC cultures were collected at 2500 × g for 2 min. The cell pellet weight was measured and analyzed for β-carotene production. The supernatant was passed through a 0.45 μm polypropylene syringe filter (Nalgene) and analyzed for β-carotene concentration.

Detection of MG1655*βC in mouse feces. Crude extracts were prepared from 0.05 g of mouse feces. These samples were used to detect the MG1655*βC transformant strain by PCR. Primers were designed to the crrB gene: crrB forward, 5‘-ACCCGCTATGTCACTCTTTGA-3’; and crrB reverse, 5‘-GTCAXTAGCCGCATCGTCAA-3’. Samples were analyzed on 1.5% agarose gel for detection of the PCR product.

Quantification of β-carotene concentration in mouse tissues. β-Carotene concentrations in serum, liver, and feces were measured by reverse-phase HPLC analysis as described previously (16). Feces were homogenized in PBS (∼0.05 g/mL feces), centrifuged (600 × g; 2 min at room temperature), and the resultant supernatant was used for the extraction following the method referenced above (16).

Efficiency of intestinal colonization. Each fecal sample was homogenized 1:10 in 1% Bacto tryptone (BD Biosciences) and diluted in the same medium. Dilutions were plated onto MacConkey agar (BD Biosciences) containing 50 mg/L Cm and 100 mg/L Str. Mucosas from the small intestine, cecum, and colon were homogenized 1:10 in 1% Bacto tryptone, diluted in the same medium, and plated onto MacConkey agar containing 50 mg/L Cm and 100 mg/L Str.

Statistical analyses. Statistical analysis was performed using SPSS Statistical Software (version 19; IBM SPSS). Normal distribution of the data was determined by the Shapiro-Wilk test, and comparisons were made among groups of normally distributed data by 1-factor ANOVA, data was determined by the Shapiro-Wilk test, and comparisons were made among groups of normally distributed data by 1-factor ANOVA, followed by post hoc analysis (least significant difference for data with homogeneous variance; Tamhane test for data with nonhomogeneous variance). Data that were not normally distributed were analyzed by the Kruskal-Wallis test, followed by the Mann-Whitney U test for individual comparisons. Statistical significance was established as \( P < 0.05 \).

Results

Generation of the E. coli MG1655* strain synthesizing β-carotene (MG1655*βC). The mouse intestine–adapted E. coli MG1655* was transformed with the pAC-BETA plasmid to generate the recombinant strain MG1655*βC. These transformed colonies were visibly yellow-orange when grown on LB agar plates (supplemented with Str and Cm) for 3 d at 37°C (Supplemental Fig. 1). MG1655*βC liquid culture preparations produced 18.6 ± 5.6 nmol of β-carotene per gram of wet cell weight (\( n = 5 \)). There was an overall consistent yield of the provitamin A carotenoid among different preparations of the same strain. β-Carotene was detectable in the culture supernatant at a concentration of 1.2 ± 0.5 pmol/L β-carotene (\( n = 5 \)). After filtration of the culture supernatant, β-carotene became undetectable, suggesting that the provitamin A carotenoid in the unfiltered supernatant was associated with residual cells, cell debris, or other insoluble materials. Thus, the majority of the β-carotene produced was cell associated. The pAC-BETA plasmid was not stably maintained in MG1655*βC in the absence of the Cm-resistance marker (data not shown).

Intestinal colonization and in situ β-carotene production by MG1655*βC in WT mice. β-Carotene–producing bacteria were detected by PCR in the feces of the WT mice under the LD-1 regimen of bacteria administration throughout the duration of the experiment (Fig. 2A). β-Carotene was detected by reverse-phase HPLC in the feces of the WT mice beginning on day 4 of the experiment. We also observed an oscillatory pattern in β-carotene concentrations, with a peak at day 8 (Fig. 2C). In addition, β-carotene was detected at low concentrations (13.0 ± 11.0 pmol/g tissue; \( n = 3 \)) in the intestinal mucosa of these mice but not in serum or liver.

Intestinal colonization and in situ β-carotene production by MG1655*βC in CMOI−/− mice. As in the WT mice, β-carotene-producing bacteria were detected by PCR in the feces of the CMOI−/− mice under the LD-1 regimen of bacteria administration throughout the duration of the experiment (Fig. 2B). β-Carotene was detected by reverse-phase HPLC in their feces (Fig. 2D). Unlike the WT mice, intact β-carotene was
detected not only in the intestinal mucosa (9.3 ± 1.9 pmol/g tissue; n = 3) but also in serum (22.3 ± 3.7 nmol/L; n = 3) and liver (96.7 ± 42.8 nmol/g; n = 3 tissue) of the CMOI<sup>−/−</sup> mice.

The effect of different regimens of administration of MG1655<sup>*-βC</sup> on β-carotene concentrations in tissues of CMOI<sup>−/−</sup> mice. Although there were trends toward differences in serum β-carotene concentrations between groups (P = 0.064), when tested individually, none were significant (Fig. 3A). There were also no significant differences in β-carotene concentrations in the liver between the different regimens (Fig. 3B). There was no significant difference in β-carotene concentrations in the feces of mice under the LD-1, HD-2, and MD treatments (Fig. 3C). However, the HD-1 treatment resulted in higher concentrations of β-carotene in feces when compared with those of mice under the LD-1 or HD-2 administration regimen (Fig. 3C). These various treatments were all effective at delivering β-carotene to the liver.

![FIGURE 3](image)

**FIGURE 3** Serum and tissue β-carotene amounts in β-carotene 15,15'-oxygense knockout (CMOI<sup>−/−</sup>) mice under various regimens of MG1655<sup>*-βC</sup> administration. Measurements by reverse-phase HPLC analysis in the serum (A), liver (B), and feces (C). CMOI<sup>−/−</sup> mice under the LD-1 and HD-1 regimens of bacteria administration were killed on day 10, whereas those on the HD-2 and MD regimens were killed on day 17 of the experiment. Values are means ± SEMs, n = 3–4 mice per group. Labeled means without a common letter indicate significant differences (P < 0.05). HD-1, high-dose 1 wk; HD-2, high-dose 2 wk; LD-1, low-dose 1 wk; MD, multi-dose.

**Efficiency of MG1655<sup>*-βC</sup> intestinal colonization in CMOI<sup>−/−</sup> mice.** Enumeration of MG1655<sup>*-βC</sup> cells in the feces of CMOI<sup>−/−</sup> mice under the HD-2 regimen showed consistent amounts of 10<sup>7</sup>–10<sup>8</sup> CFU/g feces over time (Fig. 4A). The concentrations of β-carotene in the feces also showed a similar pattern over time (Fig. 4B). In addition, the MG1655<sup>*-βC</sup> cells were also recovered from the mucosa of the same mice at concentrations of 10<sup>6</sup>–10<sup>7</sup> CFU/g from the small intestine, >10<sup>5</sup> CFU/g from the cecum, and 10<sup>3</sup>–10<sup>5</sup> CFU/g from the colon of the mice (Fig. 4C).

**Discussion**

To the best of our knowledge, this study describes the first successful attempt to generate an intestinal-adapted recombinant *E. coli* strain that synthesizes β-carotene, achieves intestinal colonization in mice, and produces β-carotene in situ, which, during intestinal absorption, is delivered to other sites within the body, including plasma and liver.

The plasmid system that we used to express β-carotene in bacteria is well established in the carotenoid field (17). The initial step in the assembly of the backbone of carotenoids involves the isomerization of a 5-carbon molecule. These 5-carbon carotenoid precursors are generated via the mevalonate pathway in bacteria (18). Cunningham et al. (12) transformed *E. coli* cells with the plasmid pAC-BETA containing the genes coding for the 4 key enzymes of the β-carotene biosynthetic pathway (*crtE, crtB, crtI*, and *crtY*) from *E. herbicola*, thus allowing β-carotene production in this strain. This same plasmid was used recently by Miller et al. (19) to engineer the probiotic *E. coli* strain Nissle 1917 to synthesize β-carotene and examine its immunostimulatory properties in vitro. This strain produced β-carotene under anaerobic conditions (reflective of the intestinal environment) and was able to activate murine dendritic cells in vitro (19). In the present study, the in vivo efficacy of a similar bacterial system to colonize the intestine, produce β-carotene, and deliver it to tissues of mice was clearly demonstrated by transforming the strain MG1655<sup>*</sup> with pAC-BETA (11). This strain is an intestine-adapted (colonizing) spontaneous mutant of *E. coli* K12 isolated following passages through streptomycin-treated mouse intestine (11). The deletion of the flagellar transcriptional regulator flhD gene in the MG1655<sup>*</sup> strain results in nonmotility and elevated expression of genes involved in carbon and energy metabolism, making this mutant strain more efficient at using carbon sources and, consequently, more able to populate the mouse intestine. A recombinant *E. coli* strain with the potential of producing β-carotene and colonizing the mouse intestine with high efficiency (MG1655<sup>*-βC</sup> strain) was obtained.

Initially, the MG1655<sup>*-βC</sup> strain was introduced into WT mice by oral dosing, after which both β-carotene and the β-carotene–producing bacteria in the feces of the mice were detected until the end of the experiment following a single administration (LD-1). This result indicates persistence of the strain in the intestine of the mice. Although β-carotene was detected in the feces and at lower concentrations in the intestine of the WT mice, it was not detected in their serum or liver. In the human intestine, CMOI cleaves dietary β-carotene with a variable efficiency that seems to be associated with a number of polymorphisms in the CMOI gene (20). Therefore, in humans, complete intestinal conversion of all of the ingested β-carotene to retinoids does not occur, and ~17–45% of the
ingested β-carotene is released into the circulation in its intact, uncleaved form (7,21). In contrast, regularly used laboratory rodents have a more active CMOI enzyme that almost completely converts β-carotene, resulting in high concentrations of β-carotene metabolites (15). As a consequence, intact β-carotene is very rarely found in serum and tissues of WT mice ingesting physiologic amounts of dietary β-carotene (6). Based on this knowledge, we inferred that, because mice display high efficiency of β-carotene-cleavage activity in the intestine (15), the β-carotene had been fully metabolized to retinoids in the mucosa of the WT mice, preventing β-carotene from reaching serum or liver.

Therefore, we performed a similar experiment with mice lacking CMOI, the main enzyme involved in the cleavage of β-carotene in vivo (5,22). The inability of these mice to cleave β-carotene in tissues makes them an optimal model for demon-
shuttle β-carotene across the plasma membrane and the bacteria wall, we hypothesize that β-carotene is released after the death of the bacteria during their physiologic life cycle within the intestine of the mammalian host. Interestingly, the β-carotene concentrations in the feces of these mice showed an oscillatory pattern, likely due to the cycle of proliferation and death of the bacteria.

Finally, although the mice in our experiments were not maintained in wire-bottom cages (to avoid coprophagy), we believe that the abovementioned oscillatory pattern of β-carotene concentrations in the feces supports our interpretation that tissue concentrations of β-carotene are the result of uptake of the provitamin A carotenoid synthesized by resident E. coli cells and not a result of coprophagy. Indeed, if the source of carotenoids was ingestion of feces containing β-carotene rather than the provitamin A from the intestine-resident bacteria, we would have expected excretion of constant concentrations of β-carotene with the feces over time.

In summary, to the best of our knowledge, this is the first report of in vivo production of β-carotene via β-carotene-producing bacteria residing in the intestine of a mammalian model and subsequent absorption and delivery to tissues of the provitamin A. Additional studies will aim at testing the efficacy of our approach in improving the vitamin A status of an appropriate mouse model of VAD.

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Literature Cited