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Auxotrophy-Based Detection of Hyperornithinemia in Mouse Blood and Urine

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Abstract

Gyrate atrophy of the choroid and retina (GACR) is a hereditary form of progressive blindness caused by homozygosity for loss-of-function mutations in the ornithine aminotransferase gene (*Oat*). The high levels of circulating ornithine that lead to ophthalmic symptoms in young adults are also displayed by 2 ornithine aminotransferase (OAT)-deficient mouse models of GACR. Here, we have developed an inexpensive and quantitative bacteria-based test for detecting hyperornithinemia in blood or urine samples from these mutant mice, a test that we suggest could be used to facilitate the identification and treatment of OAT-deficient humans before the onset of visual impairment.

Keywords

argE mutant *E coli*, gyrate atrophy of the choroid and retina, ornithine biosensor, metabolic screening, animal models of human disease

Introduction

Bacterial cells, either naturally occurring or genetically modified, have proven to be useful for detecting and quantifying various analytes in a variety of complex environments,[1-5] including amino acids in physiological fluids that might be diagnostic for certain inherited metabolic disorders.[6-8] Here we used a publically available, auxotrophic *Escherichia coli* variant to detect and quantify ornithine levels in blood or urine samples taken from mice segregating for inherited defects in ornithine aminotransferase (OAT), an enzyme which in normal adults clears excess ornithine produced in the urea cycle (see Figure 1).

These mouse mutants, the engineered *Oat* knockout[9] and the spontaneous missense variant called retarded hair growth,[10] have been shown to be excellent models for the inherited human condition known as gyrate atrophy of the choroid and retina (GACR, Online Mendelian Inheritance in Man (OMIM) #258870), which results in visual impairment that begins in childhood with myopia and night blindness and progresses to complete blindness by the fourth or fifth decade of life.[11-13] Dietary restriction of arginine has been shown to prevent (but not reverse) retinal degeneration in OAT-deficient mice.[14] In humans, the impacts of this treatment have been more varied, with results ranging from visual improvement,[15-17] marked delay of,[18-21] modest delay of,[22,23] to no discernable bearing on[24] the progression of ocular symptoms. Better outcomes in patients with GACR may correlate with earlier implementation of arginine restriction, more rigorous compliance with the dietary regimen, or both. The use of a simple and inexpensive screen for hyperornithinemia, such as we describe here, could help to identify OAT-deficient patients for treatment before retinal damage has begun. Because the biosensor system we describe is quantitative, it might also be adapted for the routine monitoring of circulating ornithine levels in patients undergoing arginine restriction.

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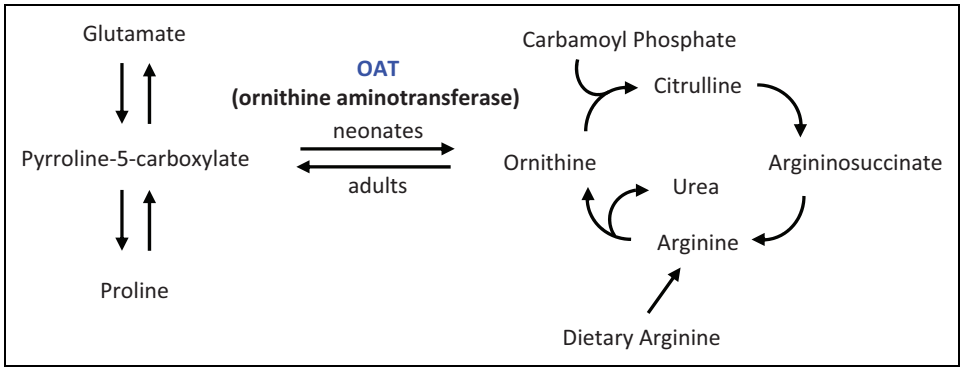

Figure 1. Pathway of arginine biosynthesis in mammals. Mutation of *Oat* (which encodes ornithine aminotransferase) blocks the synthesis of ornithine from pyrroline-5-carboxylate (P-5-C) in neonates, which is required for the synthesis of arginine in the urea cycle. In neonatal mice (but not humans), ornithine aminotransferase (OAT) deficiency leads to blood levels of arginine that are insufficient to support normal growth. In adult mice and humans, OAT deficiency blocks the conversion of ornithine to P-5-C, leading to an accumulation of ornithine in the blood that, over time, causes progressive retinal degeneration.

Methods

Mice

All animals were housed and fed according to federal guidelines, and the Institutional Animal Care and Use Committee at Central Connecticut State University (CCSU) approved all procedures involving mice (animal protocol applications #131, 134, and 136). Segregating inbred B6Ei;AKR-*Oat*^{rhg}/J mice (carrying the spontaneous retarded hair growth mutation, herein referred to as *rhg*) were obtained from The Jackson Laboratory (Bar Harbor, Maine) and are described by Bisaillon et al.[10] Mice carrying the *Oat*^{tm1Dva} targeted mutation (designated herein as *Oat*$^{\Delta}$) were kindly provided by Dr David Valle (Institute of Genetic Medicine and Departments of Pediatrics, Ophthalmology, and Molecular Biology & Genetics, The Johns Hopkins University School of Medicine, Baltimore, Maryland). The creation of the *Oat*$^{\Delta}$ mutation is described by Wang et al.[9] DNA tests for distinguishing these mutant *Oat* alleles (*Oat*$^{\Delta}$ and *rhg*) from wild type are described in Bisaillon et al.[10] Both mutant *Oat* alleles were maintained as C57BL/6J congenic stocks, and C57BL/6J mice provided our wild-type controls. *Oat*$^{\Delta}$/*Oat*$^{\Delta}$ mice, which do not survive beyond 24 hours of age without intraperitoneal injection of supplemental arginine,[9] were not available for these studies.

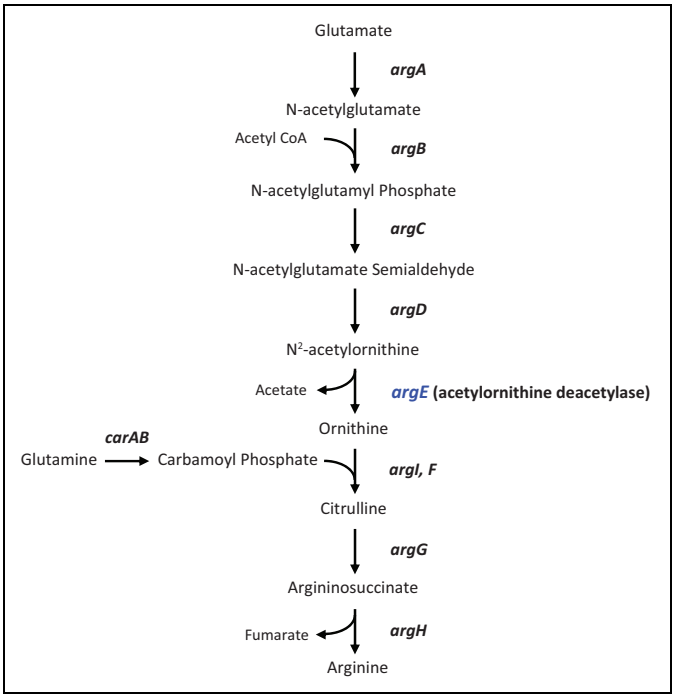

Figure 2. Pathway of arginine biosynthesis in *Escherichia coli.* Mutation of *argE* (which codes for acetylornithine deacetylase) prohibits the synthesis of ornithine, which is required for the synthesis of arginine. Growth of *argE* auxotrophs on minimal media can be rescued by supplementation with ornithine or other downstream intermediates.

Sample Preparation

Urine from 2 to 3 mice with the same genotype (+/+, +/*rhg*, +/*Oat*$^{\Delta}$, *rhg*/*rhg*, or *rhg*/*Oat*$^{\Delta}$) and gender was collected and sterilized using a 0.22-µm pore syringe filter (Thermo Fisher Scientific Inc, Waltham, Massachusetts). Samples were stored at −20°C when not used immediately. Whole blood (about 10 drops per mouse) was obtained from the facial vein using a 4-mm Goldenrod lancet (Medipoint Inc, Mineola, New York). For plasma samples, blood was collected into plasma separator Microtainer tubes with lithium heparin (Becton, Dickinson & Co, Franklin Lakes, New Jersey). For serum samples, blood was collected into 1.5 mL sterile Eppendorf tubes and allowed to clot at room temperature for 1 hour. Both blood samples were spun in a microfuge (1500*g*) at 4°C for 15 minutes to isolate plasma or serum. When not used immediately, plasma and serum samples were stored at −80°C.

The concentration of solubilized protein in mouse urine or serum was determined by a modified Bradford assay,[25] using a kit by Bio-Rad Laboratories (Hercules, California). In brief, serum was diluted 1:200 while urine was diluted 1:10 in deionized water, and 20 µL of the diluted sample was mixed with

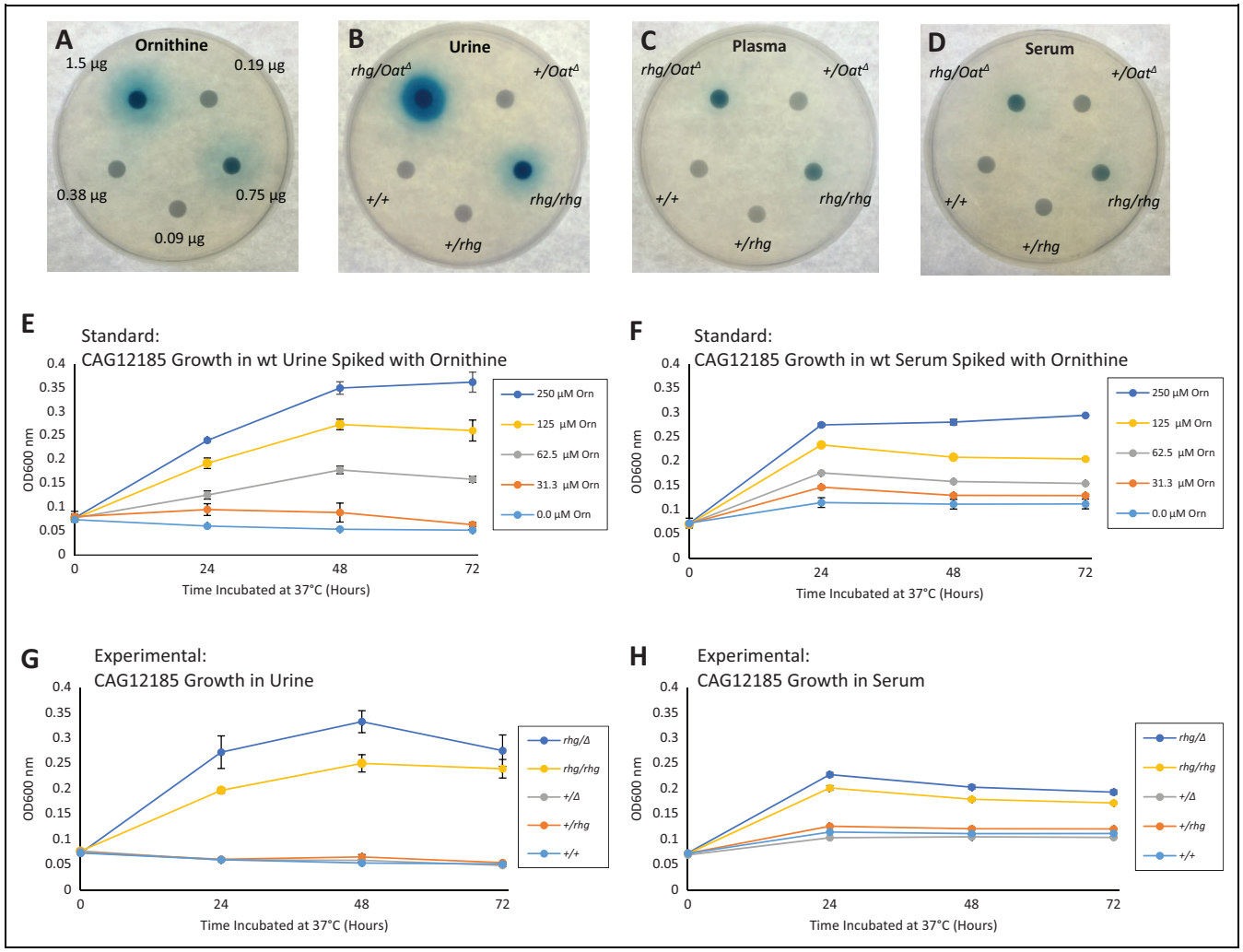

Figure 3. Rescue of the auxotrophic, *argE* mutant *Escherichia coli* strain CAG12185 on M9 minimal media by addition of exogenous ornithine or physiological fluids from mouse mutants with hyperornithinemia. A to D, Culture dishes contained M9 minimal lactose media with a top agar layer containing bacterial strain *E coli* CAG12185 and X-Gal. Sterile paper discs containing 30 µL of 5 mmol/L ornithine (resulting in 1.5 µg ornithine in the disc), 2.5 mmol/L (0.75 µg), 1.25 mmol/L (0.38 µg), 0.63 mmol/L (0.19 µg), or 0.31 mmol/L (0.09 µg) ornithine were placed on the top agar in panel A, as labeled. Ornithine diffusing from the disc in sufficient quantities can rescue the growth of auxotrophic bacteria contained within the top agar, and hydrolysis of X-Gal produces a nondiffusing blue pigment that reports the presence of accumulating bacteria. The most bacterial growth can be seen around the disc containing the 1.5 µg ornithine, which shows a more intensely colored and larger blue halo. Bacterial growth decreases with ornithine concentration until no growth can be observed around the discs containing 0.19 µg or less ornithine. In panels B to D, paper discs were loaded instead with 30 µL of: +/+, +/*rhg*, +/*Oat*$^{\Delta}$, *rhg*/*rhg*, and *rhg*/*Oat*$^{\Delta}$ urine, plasma, or serum as indicated. Growth is observed around the discs containing fluids from mutant (*rhg*/*rhg* and *rhg*/*Oat*$^{\Delta}$) mice but not around discs containing fluids from mice with at least 1 wild-type allele of *Oat*. E to H, *E coli* CAG12185 was cultured in microtiter wells that contained M9 minimal glucose media and wild-type urine (panel E) or serum (panel F) that had been spiked with various concentrations of ornithine, as indicated. In both of these "standard" assays, rescue of bacterial growth is, again, proportional to the concentration of ornithine supplied, although bacterial growth appears to be stimulated by the presence of serum, even with no ornithine added, which never stimulated growth in cultures that included urine instead of serum. In panels G and H, *E coli* CAG12185 was cultured in microtiter wells that contained M9 minimal glucose media and urine or serum, respectively, from mice with the 5 genotypes indicated. In both "experimental" assays, *rhg*/*Oat*$^{\Delta}$ urine or serum samples rescued more growth than *rhg*/*rhg* samples, but although urine from +/+, +/*rhg*, or +/*Oat*$^{\Delta}$ mice rescued no bacterial growth in these cultures, serum samples from wild-type mice with these genotypes stimulated modest increases in turbidity.

1 mL of diluted dye reagent. After incubating at room temperature for 15 minutes, 200 µL were moved to a microtiter plate where absorbance at 595 nm was recorded with a SpectraMax 250 microplate reader (Molecular Devices, LLC, Sunnyvale, California, USA) using SoftMax Pro software, version 4.7.1. Amino acid concentrations in mouse plasma were determined at Biosynthesis, Inc (Lewisville, Texas) where samples were treated with 40 g/L sulfosalicylic acid, centrifuged, and filtered to remove protein. Amino acids were separated by high-resolution ion-exchange chromatography, subjected to postcolumn ninhydrin derivatization, and detected by spectrophotometry at 570 and 440 nm. Plasma ornithine concentrations

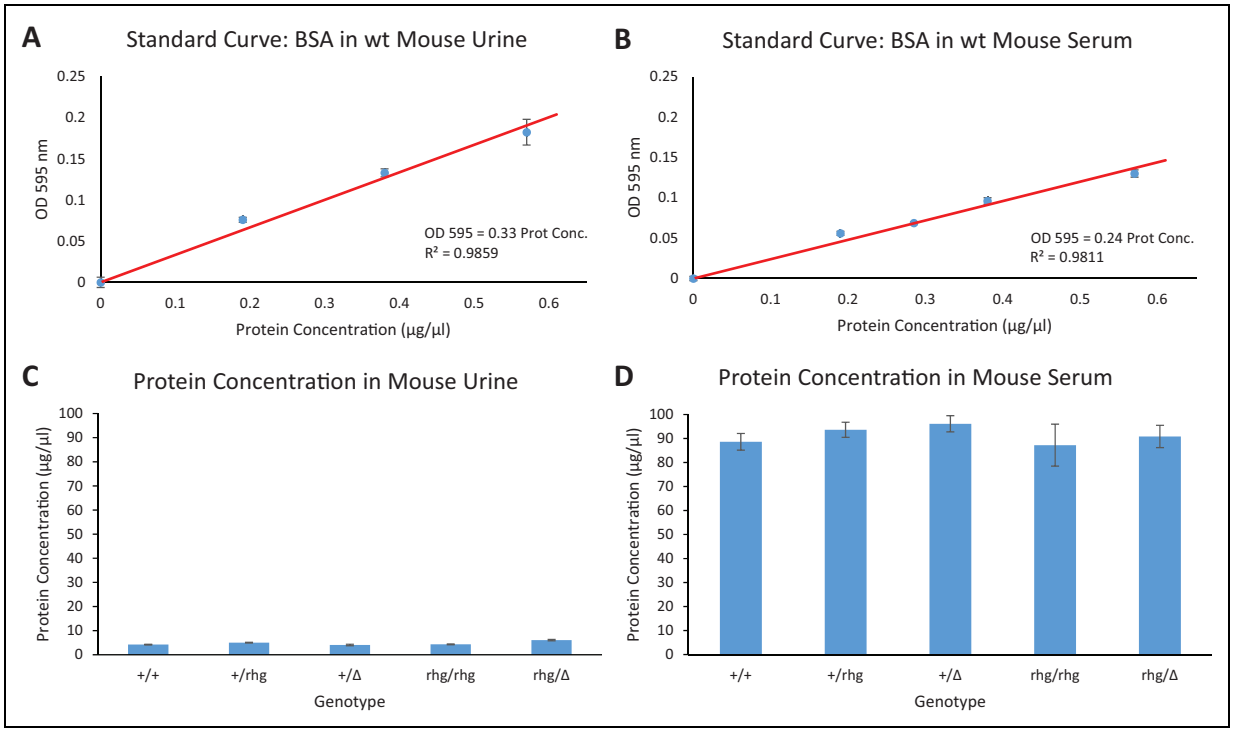

Figure 4. Bradford assay performed on mouse urine or serum to determine soluble protein concentration. A and B, Standard curves relating known protein concentration [bovine serum albumin (BSA) dissolved in wild-type mouse urine (panel A) or serum (panel B)] to absorbance at 595 nm. C, Assay performed in parallel with the standard (A) on 10-fold diluted urine samples from the 5 genotypes of mice, as indicated. All assays were performed in triplicate. Using the equation from the standard curve, the protein concentration could be determined in the overall urine sample. The range of protein concentration in urine was 4.0 to 5.7 µg/µL (an average of 4.7 ± 0.8 µg/µL). D, Assay performed in parallel with the standard (B) on 200-fold diluted serum samples from the 5 genotypes of mice, as indicated. The range of protein concentration in serum was 85.8 to 99.2 µg/µL (an average of 91.3 ± 3.6 µg/µL).

found in this survey have been reported previously by Bisaillon et al[10] and are cited again in this text for the reader's convenience; plasma arginine and citrulline concentrations are reported here for the first time.

Top Agar Bacterial Growth Assay

Escherichia coli strain CAG12185 (stock number 7471),[26] an *argE*-null mutant, was obtained from the Coli Genetic Stock Center at Yale University (New Haven, Connecticut). Glass culture tubes (16 × 125 mm) containing 2 mL of Luria broth (LB) were inoculated with CAG12185 and incubated at 37°C on a New Brunswick TC-7 roller drum, rotating at 50 to 60 rpm for 24 hours. A sample of this bacterial broth (1 mL) was then spun at 7000*g* for 1 minute to pellet the bacteria. Bacteria were washed to eliminate the LB medium by being resuspended in 1 mL of sterile saline solution (0.85% NaCl) and then centrifuged again. The bacteria were washed in this manner a total of 3 times.

M9 minimal medium,[27] supplemented with 0.2% lactose and 5 µg/mL thiamine (both from Thermo Fisher Scientific Inc), was used for top agar (0.6% agar) and plates (1.5% agar). Top agar was briefly boiled and then cooled to 50°C. Forty microliters of a 40 mg/mL stock solution of X-Gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside [Sigma-Aldrich Corp, St Louis, Missouri], dissolved in dimethylformamide) and 100 µL saline-washed bacteria were added to 4 mL of the top agar. The top agar was quickly mixed and poured onto M9 lactose plates and allowed to cool and solidify.

To sterile 6-mm-diameter filter paper discs (Cat No 231039; Becton, Dickinson & Co), 30 µL of urine, plasma, serum, or aqueous solutions of L-ornithine hydrochloride (Thermo Fisher Scientific Inc) or L-citrulline, or argininosuccinic acid, or L-arginine hydrochloride (Sigma-Aldrich Corp) were applied. The discs were allowed to air dry for 20 to 60 minutes and then applied to the top agar surface of the inoculated plates. Plates containing lawns of CAG12185 were photographed after 72 hours of incubation at 37°C.

Microtiter Plate Assays for Quantification of Ornithine Concentration in Physiological Fluids

Ninety-six–well microtiter plates (Corning Life Sciences, Cat No DL 353072, procured from Thermo Fisher Scientific Inc) were used in conjunction with a spectrophotometer to measure turbidity in wells (proportional to the concentration of bacterial cells). Wells containing a serial dilution of ornithine (250, 125, 62.5, and 31.3 µmol/L ornithine) in M9 minimal glucose media with 25 µg/mL tetracycline (Cat No T8032; Sigma-Aldrich Corp) plus 5 µL saline-washed bacteria and 7 µL of wild-type

urine or 20 µL of wild-type serum provided a standard curve for ornithine concentration versus bacterial growth in each plate assay. Wells containing M9 minimal glucose media with 25 µg/mL tetracycline plus 5 µL saline-washed bacteria and 7 µL of urine or 20 µL of wild-type serum from mice with each of 5 genotypes (+/+, +/*rhg*, +/*Oat*$^{\Delta}$, *rhg/rhg*, or *rhg/Oat*$^{\Delta}$) comprised the experimental group. A set of wells with no added *E coli* was also prepared as a control for any tetracycline-resistant bacterial contamination in the assays. Wells were also prepared with mutant bacteria in M9 minimal glucose media to ensure that any growth observed was due to ornithine rescue. All wells were created and assayed in triplicate. Microtiter plates were covered with a Breathe Easy adhesive microtiter plate seal (E&K Scientific, Inc, Santa Clara, California) and incubated shaking (30 rpm) on a rotating platform (New Brunswick Scientific Excella Model E1, procured from Thermo Fisher Scientific Inc) at 37°C for 3 days. Absorbance at 600 nm was recorded at 24-hour intervals with a SpectraMax 250 microplate reader.

Results

Top Agar Plates: Qualitative Detection of Hyperornithinemia

Escherichia coli strain CAG12185 is an *argE* mutant and therefore unable to convert glutamate into arginine (see Figure 2), a proteinogenic amino acid essential for bacterial growth. However, addition of exogenous ornithine (0.38-1.5 µg) loaded into paper discs placed on a lawn of CAG12185 can rescue growth of these auxotrophs as shown in Figure 3A. Similar results were obtained using paper discs soaked with physiological fluids (urine, plasma, or serum) from OAT-deficient mice (*rhg/rhg* and *rhg/Oat*$^{\Delta}$) that are known to accumulate excess ornithine in the blood and excrete it in the urine (Figure 3B-D). By contrast, bacterial growth around discs loaded with 0.9 to 0.19 µg ornithine or saturated with physiological fluids from phenotypically wild-type mice (+/+, +/*rhg*, or +/*Oat*$^{\Delta}$) was not detectable in these top agar assays. Thus, this simple biosensing method can readily distinguish subjects with hyperornithinemia from controls with 1 or 2 wild-type *Oat* alleles.

Microtiter Plates: Quantitative Detection of Hyperornithinemia

To better quantify the extent of bacterial growth in response to various levels of exogenous ornithine, bacterial samples were next grown in liquid M9 minimal media inside the wells of microtiter plates. For the first round of these assays, wild-type (+/+) urine or serum samples were spiked with known concentrations of ornithine (250, 125, 62.5, or 31.3 µmol/L ornithine), and bacterial growth (expressed as turbidity) was measured as absorbance at 600 nm at 24-hour intervals over 3 days. The resulting graphs show that bacterial growth increases in proportion to the concentration of added ornithine (see Figure 3E and F). It is notable that even with no ornithine added to the serum, bacterial growth was rescued by 24 hours,

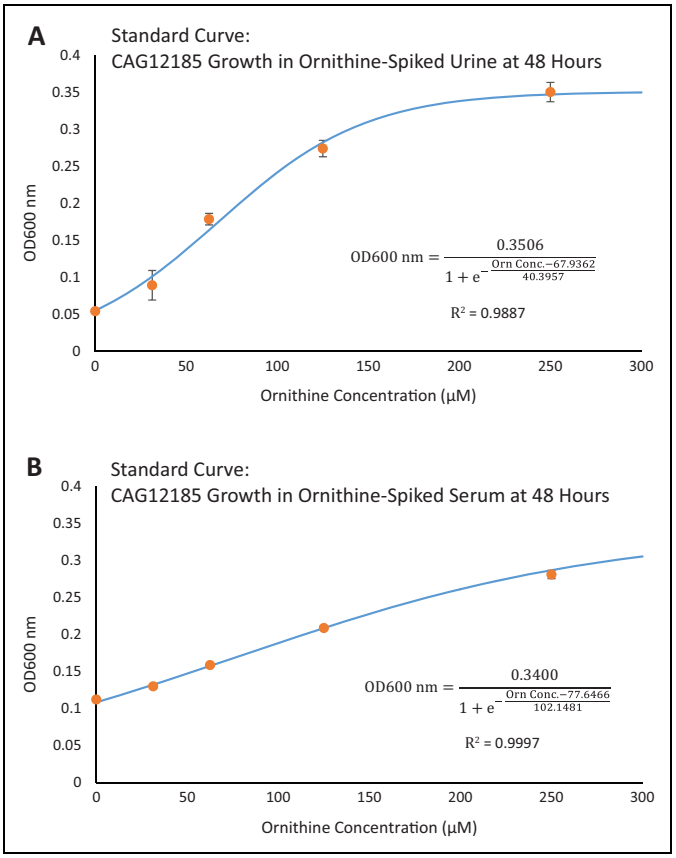

Figure 5. Standard curves relating the growth of *Escherichia coli* strain CAG12185 to the known concentration of ornithine added to cultures containing wild-type mouse urine (panel A) or serum (panel B). These standard curves correspond to the standard assays shown in Figure 3, panels E and F. Turbidity in the culture wells at 48 hours of incubation (indicative of bacterial growth and based on the average of 3 measures of absorbance at 600 nm) was plotted against the known concentrations of ornithine present in the microtiter well. A 3-parameter sigmoidal logistic regression was generated from the plotted values to produce an equation relating ornithine concentration to growth. From the equation and the absorbance readings resulting from bacterial growth in the physiological fluid samples gathered from mutant *rhg/rhg* or *rhg/Oat*$^{\Delta}$ mice, ornithine concentrations in the respective physiological fluid samples were determined (see text).

something never seen in the microplate assays of urine. We think this is likely due to the higher level of solubilized protein found in serum (measured here to be 91 ± 4 µg/µL) compared to urine (measured here to be 4.7 ± 0.8 µg/µL; see Figure 4) or amino acid monomers (such as ornithine, citrulline, and arginine; measured here to be 77.4 ± 2.2 µmol/L, 99.3 ± 5.6 µmol/L, and 122 ± 15 µmol/L, respectively, in wild-type mouse plasma [N = 12]), which might provide the auxotrophic mutants with an exogenous source of arginine.

Finally, bacterial samples were grown in microtiter plate wells that included urine or serum samples from mice with 5 different genotypes (+/+, +/*rhg*, +/*Oat*$^{\Delta}$, *rhg/rhg*, or *rhg/Oat*$^{\Delta}$) added to M9 minimal media (but with no added ornithine). In assays of urine, *rhg/Oat*$^{\Delta}$ samples rescued more growth than *rhg/rhg* samples, and wild-type samples rescued

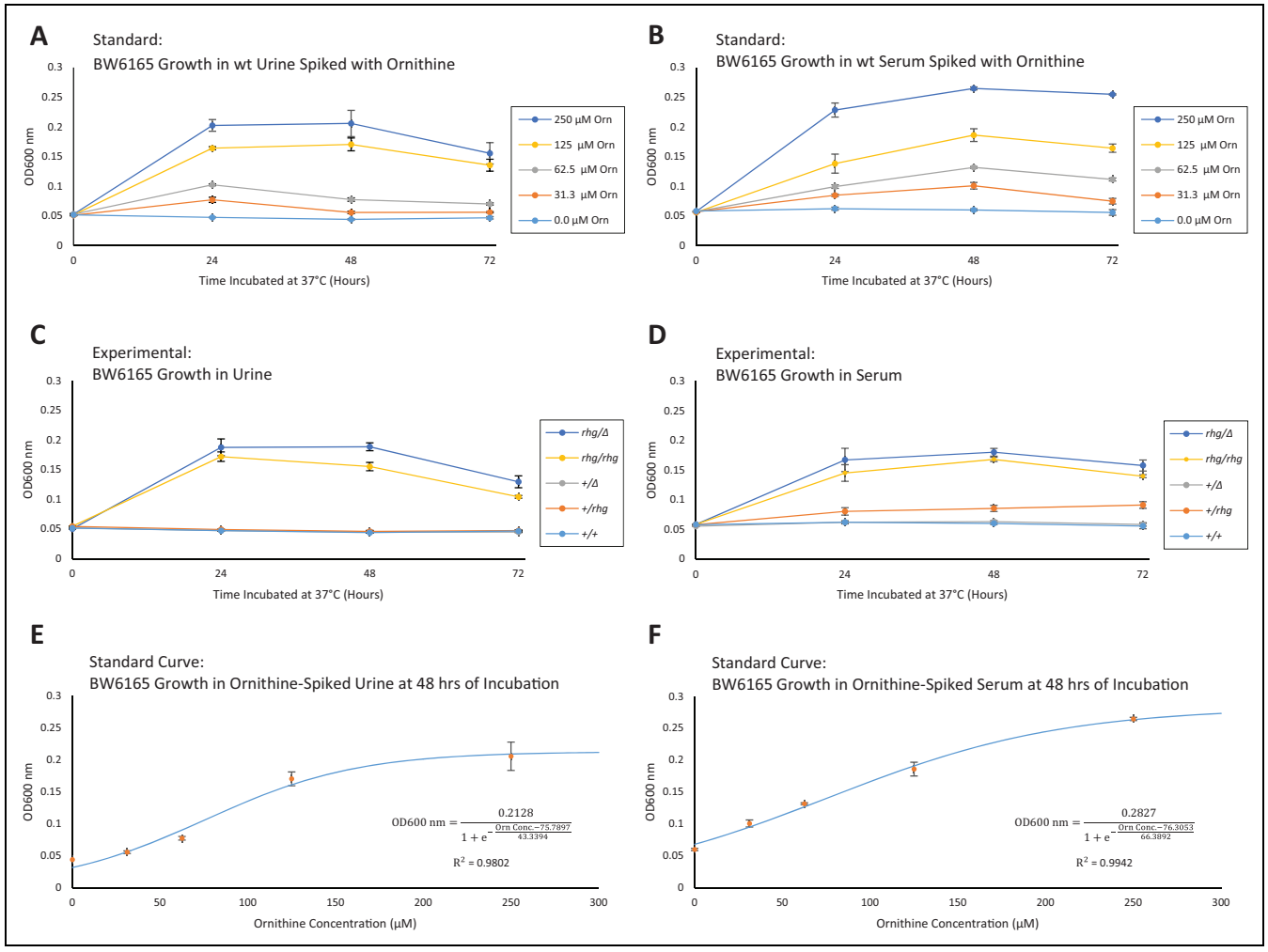

Figure 6. Rescue of the auxotrophic, *argE* mutant *Escherichia coli* strain BW6165 on M9 minimal media by addition of exogenous ornithine or physiological fluids from mouse mutants with hyperornithinemia. A and B, *E coli* BW6165 was cultured in microtiter wells that contained M9 minimal glucose media and wild-type urine (panel A) or serum (panel B) that had been spiked with various concentrations of ornithine, as indicated. In both standard assays, rescue of bacterial growth was proportional to the concentration of ornithine supplied. In panels C and D, *E coli* BW6165 was cultured in microtiter wells that contained M9 minimal glucose media and urine or serum, respectively, from mice with the 5 genotypes indicated. In both experimental assays, *rhg*/*Oat*$^{\Delta}$ urine or serum samples rescued more growth than *rhg*/*rhg* samples, but although urine from +/+, +/*rhg*, or +/*Oat*$^{\Delta}$ mice rescued no bacterial growth in these cultures, serum samples from wild-type mice with these genotypes sometimes stimulated modest increases in turbidity (eg, see +/*rhg* culture in panel D). Similar results were obtained in microtiter culture experiments that used *E coli* strain CAG12185 (see Figure 3). E and F, Standard curves relating the growth of *E coli* strain BW6165 to known concentrations of ornithine added in wild-type mouse urine (panel E) or serum (panel F), based on the average of 3 turbidity readings taken at 48 hours of incubation in the same assays shown in panels A and B, respectively. A 3-parameter sigmoidal logistic regression was generated from the plotted values to produce an equation relating ornithine concentration to growth. With this equation, bacterial growth observed in the experimental assays of mutant (*rhg*/*rhg* or *rhg*/*Oat*$^{\Delta}$) urine (panel C) or serum (panel D) can be interpreted as ornithine concentrations in the respective physiological fluid samples. From the standard curve shown in panel E, the ornithine concentration in urine from mutant *rhg*/*rhg* mice was 3.4 $\pm$ 0.2 mmol/L (N = 3) and from *rhg*/*Oat*$^{\Delta}$ mice was 4.7 $\pm$ 0.4 mmol/L (N = 3), in close agreement with values determined using *E coli* strain CAG12185 (see text). From the standard curve shown in panel F, the ornithine concentrations in mutant serum samples were found to be 1015 $\pm$ 35 µmol/L for *rhg*/*rhg* (N = 3) and 1139 $\pm$ 69 µmol/L for *rhg*/*Oat*$^{\Delta}$ serum (N = 3), again in good agreement with values determined using *E coli* CAG12185 and with values measured directly by Bisaillon et al[10] (see text).

no growth (Figure 3G). In assays of serum, similar results were obtained (see Figure 3H), but in these assays, the wild-type samples (+/+, +/*rhg*, and +/*Oat*$^{\Delta}$) routinely showed a slight increase in absorbance over time (indicating growth), which never occurred in urine-based assays. Although, again, we think this is likely due to higher concentrations of solubilized protein or amino acids found in serum (compared with urine), the level of bacterial growth observed in mutant (*rhg*/*rhg* and *rhg*/*Oat*$^{\Delta}$) serum samples is nonetheless distinctly higher than in wild-type samples, presumably due to hyperornithinemia.

Bacterial growth in microtiter well cultures that include experimental urine or serum samples can be compared to the

growth induced with known amounts of ornithine to infer the ornithine concentration in the experimental samples. For example, if we use absorbance at 48 hours to generate a sigmoidal logistic regression curve (see Figure 5B), fitting absorbance at 48 hours in *rhg/rhg* or *rhg/Oat*$^{\Delta}$ serum assays to that curve indicates 892 $\pm$ 36 μmol/L ornithine in *rhg/rhg* serum (N = 3) and 1192 $\pm$ 51 μmol/L in *rhg/Oat*$^{\Delta}$ serum (N = 3). These concentrations are in good agreement with the plasma ornithine concentrations obtained by direct measurement in the study by Bisaillon et al,[10] which were 1046 $\pm$ 109 μmol/L for *rhg/rhg* (N = 6) and 1244 $\pm$ 344 μmol/L for *rhg/Oat*$^{\Delta}$ (N = 6). Ornithine concentrations determined by this approach for urine samples (4.7 $\pm$ 0.2 mmol/L ornithine in *rhg/Oat*$^{\Delta}$ mouse urine and 3.0 $\pm$ 0.3 mmol/L in the *rhg/rhg* mouse urine; see Figure 5A) were more dramatically elevated versus wild-type samples than were those determined using serum samples (and are thus more easily detected by this assay), but these values for ornithine concentration in urine are difficult to compare with measures made by other methods, which are typically normalized in relation to the amount of citrulline excreted in the same sample, which was not determined here.

Discussion

We have shown that the auxotrophic CAG12185 strain of *E coli* makes an effective biosensor for the rapid and inexpensive detection of ornithine in liquid media including mouse urine and blood. Because the growth of this auxotroph is proportional to the amount of ornithine in the media, the ornithine concentration in the experimental samples can be estimated by comparison with bacterial growth in similar solutions with known ornithine concentrations. Indeed, the levels of ornithine measured by this approach in mutant mouse blood were comparable to levels previously found for these subjects by direct chemical analysis.[9,10] Similar results were obtained in microtiter culture experiments that used *E coli* strain BW6165 (Coli Genetic Stock Center, Stock Number 6760, Yale University)[28] instead of CAG12185 (see Figure 6), but this *lacY* mutant strain yielded inconsistent results in top agar assays (data not shown).

Because reduction in dietary arginine has been shown to slow the progress of retinal degeneration in most patients—especially those who start arginine restriction before the onset of visual symptoms—we suggest that the simple and inexpensive test we describe here for the detection of hyperornithinemia might be used to screen young children from families affected by GACR (or even larger populations) to help improve the efficacy of this proven treatment. In addition to early institution of the highly restrictive diet, rigorous compliance is also required to successfully maintain, over the long term, a chronic reduction in plasma ornithine. Although our assays were not able to detect blood ornithine at concentrations near normal physiological levels (about 40-120 μmol/L[9,20]), blood levels under 400 to 500 μmol/L are generally considered therapeutic.[20] Thus, our method, as described, appears to provide a range of sensitivity that would allow patients with GACR to routinely monitor their blood levels of ornithine, facilitating their better maintenance of optimum biochemical control. A second, though more rare, hyperornithinemia, known as hyperornithinemia–hyperammonemia–homocitrullinuria syndrome (OMIM #238970), results from homozygous defects in the mitochondrial ornithine transporter encoded by *Slc25a15* and can present plasma ornithine levels in the range of 216 to 1915 μmol/L.[29] The developmental delays and neurologic symptoms of this disorder can be prevented by a low-protein diet initiated early in life,[30] so early detection by a test like we describe might lead to more favorable outcomes.

Because growth of the auxotrophic mutants we used here can also be rescued by the addition of arginine, argininosuccinate, or citrulline to minimal media cultures (data not shown), this method might also screen for metabolic disturbances caused by other enzymatic defects (eg, ornithine transcarbamylase deficiency, OMIM #311250; argininosuccinic acid synthetase deficiency, #215700; argininosuccinic acid lyase deficiency, #207900; or arginase deficiency, #207800) that could lead to excess levels of these urea cycle constituents in physiological fluids. To refine diagnoses, perhaps testing a small panel of single-gene auxotrophs (such as *argF*, *argG*, or *argH* loss-of-function variants; see Figure 2) for growth rescue on minimal media by physiological fluids in conjunction with *argE* mutants (or as a follow-up for growth-positive samples) might be informative. Animal models for these urea cycle disorders[31-36] might be helpful in testing and refining such a screening strategy.

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Authors' Note

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Declaration of Conflicting Interests

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