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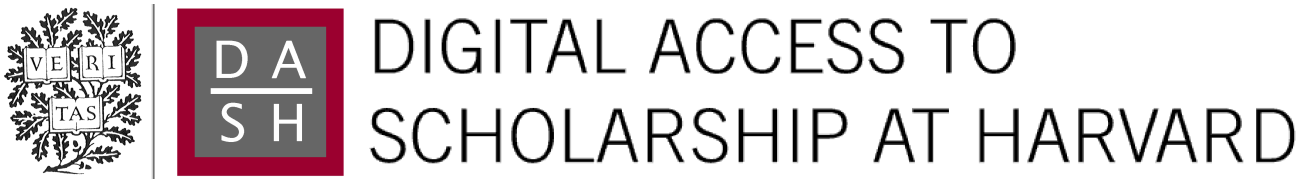

Predicting and Manipulating Cardiac Drug Inactivation by the Human Gut Bacterium Eggerthella lenta

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(Article begins on next page)

Title: Predicting and manipulating cardiac drug inactivation by the human gut bacterium *Eggerthella lenta*

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Abstract:

Despite numerous examples of the effects of the human gastrointestinal microbiome on drug efficacy and toxicity, there is often an incomplete understanding of the underlying mechanisms. Here, we dissect the inactivation of the cardiac drug digoxin by the gut Actinobacterium *Eggerthella lenta*. Transcriptional profiling, comparative genomics, and culture-based assays revealed a cytochrome-encoding operon up-regulated by digoxin, inhibited by arginine, absent in non-metabolizing *E. lenta* strains, and predictive of digoxin inactivation by the human gut microbiome. Pharmacokinetic studies using gnotobiotic mice revealed that dietary protein reduces the *in vivo* microbial metabolism of digoxin, with significant changes to drug concentration in the serum and urine. These results emphasize the importance of viewing pharmacology from the perspective of both our human and microbial genomes.

One Sentence Summary:

A microbial biomarker predicts digoxin inactivation by the human gut microbiome; studies in mice show that dietary protein prevents this biotransformation.

Main Text:

Humans are home to large and diverse microbial communities, the most abundant of which resides in the gastrointestinal tract. Recent studies have highlighted the clinical relevance of the biotransformations catalyzed by the human gut microbiome, including alterations to the bioavailability, activity, and toxicity of therapeutic drugs (*1, 2*). Although >40 drugs are metabolized by the gut microbiome, little is known about the underlying mechanisms. This knowledge is critical to enable the rational design of pharmaceutical or dietary interventions.

The inactivation of the cardiac drug digoxin provides a promising starting point for understanding microbial drug metabolism. Digoxin and other cardiac glycosides have been widely used for hundreds of years to treat heart failure and arrhythmias. Therapeutic effects are accomplished indirectly when inhibition of the Na^+/K^+ ATPase in cardiac myocytes raises the intracellular Ca^{2+} concentration (*3*). Digoxin has a narrow therapeutic range (0.5-2.0 ng/mL) (*3*), and some patients excrete the inactive digoxin metabolite, dihydrodigoxin, in which the lactone ring is reduced (**fig. S1A**) (*4*). This modification disrupts ring planarity, which is thought to shift

positioning within the binding pocket of the Na^+/K^+ ATPase, resulting in decreased target affinity (*5*). Co-administration of broad spectrum antibiotics increases serum digoxin (*4*), and *Eggerthella lenta* reduces digoxin *in vitro* (*6*). Prior to this work, the molecular mechanism of digoxin reduction and the factors that alter microbial drug inactivation *in vivo* were unknown.

We confirmed that *E. lenta* DSM2243, the type strain, reduces digoxin *in vitro (7)*, and that arginine inhibits this reaction (**Fig. 1A**). The growth of *E. lenta* DSM2243 was stimulated by arginine supplementation (**Fig. 1A,S2**), indicative of using the arginine dihydrolase pathway for ATP (*8*). Citrulline (an intermediate upstream of ATP production) stimulated growth, whereas ornithine (an end product) did not (**figs. S2,S3**).

E. lenta cultures were grown anaerobically in rich medium supplemented with low- and high-levels of arginine (0.25% and 1.25%, respectively) in the presence or absence of digoxin (10 μg/mL) and we performed RNA-Seq on the resultant cellular biomass (**figs. S4-6, table S1**). A two-gene operon was highly up-regulated after exposure to digoxin during exponential growth (>100-fold; **Fig. 1B, tables S2,S3**). These two genes, referred to here as the cardiac glycoside reductase (*cgr*) operon (gene labels: *cgr1* and *cgr2*), encode proteins that are homologous to bacterial cytochromes and are therefore potentially capable of using digoxin as an alternative electron acceptor. Incubation of *E. lenta* with multiple cardiac glycosides and their reduced forms revealed that the *cgr* operon is broadly responsive to compounds with an α,β-unsaturated butyrolactone ring (**figs. S7-9, table S5**).

Digoxin induction was increased in low arginine conditions during both exponential and stationary phase, relative to cultures exposed to high levels of arginine (**fig. S10A,B**). *cgr* induction by digoxin, and the growth phase-dependent effects exerted by arginine were confirmed on independent samples using qRT-PCR (**Figs. 1C,S7C, table S4**). Unlike arginine, ornithine did not repress *cgr2* expression (**fig. S11**). These results are consistent with the hypothesis that arginine represses *cgr* operon expression, thereby inhibiting digoxin reduction.

Next, we tested three strains of *E. lenta* (DSM2243, FAA 1-3-56, and FAA 1-1-60) (*9, 10*), for digoxin reduction; the type strain was the sole strain capable of digoxin reduction *in vitro* (**Fig. 1D)**. Comparative genomics revealed that the type strain was nearly indistinguishable from the other two strains using common marker genes (**fig. S12**). Reciprocal BLASTP comparisons of all protein-coding sequences of the three fully sequenced *E. lenta* strains revealed that the type strain shared 79.4% and 90.5% of its proteome with strains FAA 1-3-56

and FAA 1-1-60, respectively (**fig. S12**). The *cgr* operon was unique to the type strain (**table S6**); furthermore, the two non-reducing *E. lenta* strains were missing three genomic loci which were also up-regulated by digoxin, and are predicted to encode membrane transporters for the uptake of small molecules and glycosides (**fig. S13**). Arginine did not significantly decrease the expression level of these transporters (**fig. S14**).

Strain-level variation provides an explanation for the difficulties in predicting dihydrodigoxin levels in cardiac patients by the presence or absence of *E. lenta* (*6, 11*). We used qPCR to measure the relative abundance of the *cgr* operon to the *E. lenta* 16S rRNA gene (the "*cgr* ratio") in microbial community DNA from 20 unrelated healthy people, along with *ex vivo* digoxin reduction assays. The results stratified our cohort into low reducers (12.82±10.68% reduction; n=6) and high reducers (96.25±7.69% reduction; n=14) (**Fig. 2A**). The *cgr* ratio was significantly increased for the high reducers (1.058±0.562) when compared to low reducers (0.425±0.582; $P<0.05$, Student's *t* test) (**Fig. 2B,S15**). Linear regression of reduction efficiency with the *cgr* ratio revealed a significant correlation ($R^2=0.22$, $P<0.05$), whereas the abundance of *E. lenta* failed to predict the extent of reduction ($R^2=0.06$, $P=0.30$). The optimal *cgr* ratio cutoff (0.6) predicted digoxin reduction efficiency with a sensitivity of 86%, specificity of 83%, and precision of 92%.

Co-culture of *E. lenta* with the fecal microbiome enhanced the efficiency of digoxin reduction. Each low-reducing fecal samples was incubated with the type (reducing) and FAA 1-3-56 (non-reducing) strains of *E. lenta*. The communities incubated with the type strain reduced more digoxin (95.39±2.41%) than the type strain alone (68.91±7.70%; $P<0.05$, Mann-Whitney test) (**Fig. 2C**). The *cgr* ratio was significantly elevated after co-culture (**Fig. 2D**), and was tightly linked to reduction efficiency ($R^2=0.74$, $P<0.0001$). An explanation for the observed microbial synergy is that the fastidious growth of *E. lenta* is promoted by growth factors supplied by the gut microbiota, a phenomena that is known to impact the metabolism of environmental pollutants by soil microbial communities (*12*), along with competition for arginine that boosts digoxin reduction by *E. lenta*. Consistent with these hypotheses, the abundance of the *E. lenta* type strain was significantly increased in the presence of a complex microbial community (1.6e6±4.8e5 vs. 1.8e5±8.4e3 in isolation; $P<0.05$, Mann-Whitney test), and arginine supplementation suppressed the reduction of digoxin during co-culture (**fig. S16**).

Diet could also explain inter-individual variations in digoxin reduction. *In vitro* growth of *E. lenta* showed that while arginine stimulated cell growth, it decreased *cgr* operon expression, and prevented the conversion of digoxin to dihydrodigoxin (**Figs. 1A,C,S10**). These observations led us to hypothesize that increased consumption of dietary protein, and the corresponding increase in arginine, would inhibit the *in vivo* reduction of digoxin by *E. lenta*. Germ-free adult male Swiss-Webster mice were colonized with the type strain prior to being fed diets differing only in the amount of total protein (n=5 mice/group; **tables S7,S8**; **fig. S17A**). *E. lenta* colonized mice on both diets (**fig. S18A**), and exhibited high levels of expression of the *cgr* operon (**fig. S18B**). Quantification of serum and urine digoxin (*7*) revealed significant increases on the high protein diet, indicative of suppressed digoxin reduction by *E. lenta* (**Fig. 3A,B**). These trends were also consistent with fecal analysis of samples from each group of mice 4-16 hours following digoxin administration (**Fig. 3**). We also confirmed that the high protein diet significantly elevated the level of amino acids in the distal small intestine (*7*), resulting in a fold increase of 1.71±0.06 ($p<0.001$, Wilcoxon test; **tables S9,10)**.

We controlled for the indirect effects of host diet and colonization that might alter digoxin pharmacokinetics irrespective of reduction by *E. lenta*. Germ-free mice were colonized with either the digoxin-reducing type strain or the non-reducing FAA 1-3-56 strain, and subsequently fed the same two diets (**fig. S17B**). As seen before, we detected colonization with both strains, high *cgr* operon expression, and elevated serum and urine digoxin on the high protein diet for mice colonized with the type strain (**Figs. 3C,D,S18C,D**). Diet did not significantly impact the serum or urine digoxin levels of mice colonized with the non-reducing strain (**Fig. 3C,D).** Serum digoxin was significantly lower in mice colonized with the type strain on the 0% protein diet, relative to those colonized with the non-reducing strain (4.91±1.56 ng/mL vs. 13.8±1.25; $P<0.01$, Student's *t* test, **Fig. 3C**). Together, these results suggest that the increased level of free amino acids available to *E. lenta* inhibited the activity of the *cgr* operon, increasing the bioavailability of digoxin.

An expanded model of digoxin pharmacokinetics is now emerging: colonization by distinct strains of *E. lenta,* microbial interactions, and host diet act together to influence drug levels (**fig. S19**). Follow-up studies in cardiac patients are necessary to determine if rapid qPCR-based biomarker assessments of the gut microbiome can guide dosage regimes. It may also be possible to provide dietary guidelines or supplements that prevent microbial drug metabolism.

More broadly, our results emphasize that a comprehensive view of pharmacology includes the structure and activity of our resident microbial communities, and a deeper understanding of their interactions with each other, with their host habitat, and with the nutritional milieu of the gastrointestinal tract.

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Figure legends

Fig. 1. Discovery of a bacterial operon induced by digoxin. (A) Arginine stimulates the growth of *E. lenta* DSM2243 *in vitro* while blocking the reduction of digoxin. Maximum OD_{600} (solid line; values are the mean±sem; n=3) and digoxin % reduction efficiency (dashed line; values are the mean; n=2) after 48 hours of growth. (B) RNA-Seq profiles of the cardiac glycoside reductase (*cgr*) operon are shown with/without digoxin during exponential growth in medium containing low/high arginine. The height is proportional to the natural log of the number of unambiguous sequencing reads mapped to each base. (C) *cgr2* transcription as determined by qRT-PCR. Asterisks indicate statistical significance by Student's *t* test ($P<0.05$). Horizontal lines are the mean; n=2-3. (D) Identification of 2 strains of *E. lenta* incapable of reducing digoxin. Values are the mean±sem; n=3. ND=no reduction detected.

Fig. 2. A microbial biomarker predicts the inactivation of digoxin. (A) Liquid chromatography/mass spectrometry (LC/MS) was used to quantify digoxin reduction in the fecal microbiomes of 20 unrelated individuals. (B) The *cgr* ratio was significantly different between low and high reducers. Data represent qPCR using the *cgr2* gene, and *E. lenta* specific 16S rDNA primers (**table S4**). (C) Five low reducing fecal microbial communities were incubated for five days in the presence or absence of *E. lenta* DSM2243 or FAA 1-3-56. LC/MS was used to quantify the completion of digoxin reduction. Supplementation with the non-reducing strain of *E. lenta* did not significantly affect digoxin reduction efficiency. (D) The *cgr* ratio was obtained for each of the low reducing microbial communities post incubation. Outliers were identified using Grubbs' test ($P<0.01$) and removed. Values are the mean±sem. Points in A,B represent biological replicates. Asterisks indicate statistical significance by Student's *t* test (*=$P<0.05$; ***=$P<0.001$; ****=$P<0.0001$).

Fig. 3. Dietary protein blocks the inactivation of digoxin. Serum (A) and urinary (B) digoxin levels from the type strain experiment. Fecal digoxin levels showed a consistent trend: the mean area under the curve was 6.226 ng digoxin*h/mL in germ-free mice, 3.576 for mice on the 0% protein diet, and 6.364 for mice on the 20% protein diet. Serum (C) and urinary (D) digoxin levels from each group. Digoxin levels were quantified by ELISA (*7*). Values are the mean±sem.

Asterisks indicate statistical significance by Student's *t* test (*=P<0.05; **=P<0.01). n=4-5 mice/group. NS=not significant.