Bacterial deamination of residual levodopa medication for Parkinson's disease elicits inhibitory effect on gut motility

Authors: Sebastiaan P. van Kessel[1], Hiltje R. de Jong[1], Simon L. Winkel[1], Sander S. van Leeuwen[1†], Sieger A. Nelemans[2], Hjalmar Permentier[3], Ali Keshavarzian[4], Sahar El Aidy[1*]

Affiliations:

[1] Department of Molecular Immunology and Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Nijenborgh 7, 9747 AG Groningen, The Netherlands.

[2] Department of Molecular Neurobiology, Groningen Institute for Evolutionary Life Sciences (GELIFES), University of Groningen, Nijenborgh 7, 9747 AG Groningen, The Netherlands.

[3] Interfaculty Mass Spectrometry Center, University of Groningen, The Netherlands.

[4] Division of Digestive Disease and Nutrition, Section of Gastroenterology, Department of Internal Medicine, Rush University Medical Center, 1725 W. Harrison, Suite 206, Chicago, Illinois 60612, USA.

[†] Current address: Department of Laboratory Medicine, Cluster Human Nutrition & Health, University Medical Center Groningen (UMCG), Hanzeplein 1, 9713 GZ, Groningen, The Netherlands

[*] Corresponding author. Email: sahar.elaidy@rug.nl

Address: Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Nijenborg 7, 9747 AG Groningen, The Netherlands. P: +31(0)503632201.

Abstract

Parkinson's disease is associated with gastrointestinal tract dysfunction, where constipation is reported as the most common gastrointestinal symptom. Aromatic bacterial metabolites are attracting considerable attention due to their implication on gut homeostasis and host's physiology. *Clostridium sporogenes* is a key contributor to the production of these bioactive metabolites in the human gut. Here, we show that *C. sporogenes* effectively deaminate non-proteinogenic aromatic amino acids, such as levodopa, the main treatment in Parkinson's disease, and identify the aromatic aminotransferase responsible for the deamination. The deaminated metabolite from levodopa, 3-(3,4-dihydroxyphenyl) propionic acid, is detected in fecal samples of Parkinson's disease patients on levodopa medication, elicits an inhibitory effect on gut motility. Overall, this study underpins the importance of the metabolic pathways of the gut microbiome involved in drug metabolism not only to preserve drug effectiveness, but also to avoid potential side effects of bacterial breakdown products of the unabsorbed residue of medication.

38 **Introduction**

39 Gut bacteria produce a wide range of small bioactive molecules from different chemical classes,
40 including aromatic amino acids (Donia and Fischbach, 2015). Bacterial products from aromatic amino
41 acid degradation have been shown to play a critical role in intestinal barrier function, immune
42 modulation and gut motility (Bansal et al., 2010; Bhattarai et al., 2018; Schiering et al., 2017; Venkatesh
43 et al., 2014; Yano et al., 2015). In the lower part of the gastrointestinal tract, where oxygen is limited,
44 aromatic amino acid degradation by anaerobic bacteria involves reductive or oxidative deamination
45 (Barker, 1981) resulting in production of aromatic metabolites (Dodd et al., 2017; Elsden et al., 1976;
46 Nierop Groot and De Bont, 1998; Yvon et al., 1997). Among these metabolites is 3-indolepropionic acid
47 (IPA), which is produced from tryptophan deamination by *Clostridium sporogenes* (Hoogerheide and
48 Kocholaty, 1938), via tryptophan transaminase (O'Neil and DeMoss, 1968) and indolelactate
49 dehydrogenase (Jean and DeMoss, 1968). We hypothesized that the non-proteinogenic amino acid and
50 the main treatment of Parkinson's disease (PD), levodopa, could also be deaminated by the gut
51 bacterium *C. sporogenes.* More recently, the enzymes involved in the deamination pathway of the
52 aromatic amino acids tryptophan, phenylalanine and tyrosine were described (Dickert et al., 2000, 2002;
53 Dodd et al., 2017). However, the enzyme involved in the initial transamination step remains unknown.

54 Recently, small intestinal microbiota have been implicated in the interference with levodopa drug
55 availability (van Kessel et al., 2019; Maini Rekdal et al., 2019). Early *in vivo* studies showed that ~90%
56 of levodopa is transported to the circulatory system (Bianchine et al., 1972; Morgan, 1971; Sasahara et
57 al., 1981), leaving a ~10% non-absorbed fraction of residual levodopa that can act as substrate for other
58 bacterial species associated with the lower, more anaerobic regions of the GI-tract (Goldin et al., 1973).
59 Such bacterial-residual drug interaction might act as bioactive metabolites with an impact on gut
60 homeostasis.

61 PD is often associated with non-motor symptoms especially of the gastrointestinal (GI) tract. GI-tract
62 dysfunction such as constipation, drooling and swallowing disorders occur frequently in PD patients,
63 especially constipation, which is reported in 80-90% of the PD patients (Fasano et al., 2015).
64 Importantly, chronic idiopathic constipation is associated with small intestinal (SI) motor abnormalities

65 in the esophagus, stomach, jejunum and ileum (Panagamuwa et al., 1994; Van Der Sijp et al., 1993) and
66 patients with constipation have a longer SI transit time compared to controls (Van Der Sijp et al., 1993).
67 Only recently, SI dysfunction in PD was studied observing that the transit time in the SI was significantly
68 longer in PD patients compared to healthy controls (Dutkiewicz et al., 2015; Knudsen et al., 2017).
69 Using wireless electromagnetic capsules, the median small intestinal transit time observed was
70 significantly increased between PD patients (400 min; n=22) and healthy controls (295 min, n=15)
71 (Knudsen et al., 2017).

72 This study unravels the aminotransferase responsible for initiating the deamination pathway involved in
73 the transamination of (among others) levodopa and shows that *C. sporogenes* can effectively deaminate
74 levodopa to 3-(3,4-dihydroxyphenyl) propionic acid through the same pathway as the other aromatic
75 amino acids. We show that the deamination product of gut bacterial degradation of the unabsorbed
76 residues of levodopa in fecal samples from PD patients impacts intestinal motility *ex-vivo*, highlighting
77 the urgency for further research on the effects of bacterial conversion of the unabsorbed residues of
78 medication, which might affect the host physiology.

79 **Results**

80 ***Clostridium sporogenes* deaminates levodopa**

81 *C. sporogenes* is able to deaminate proteinogenic aromatic amino acids (PAAA) through an anaerobic
82 deamination pathway. We hypothesized that levodopa, a non-proteinogenic amino acid (NPAAA) and
83 the main treatment in PD might be deaminated through the same pathway. To this end, cultures were
84 incubated with 100 µMµM levodopa or 5-hydroxytryptophan (another NPAAA and precursor of
85 serotonin) in combination with PAAAs from the growth medium and followed over time. Analysis of
86 the samples using High Pressure Liquid Chromatography (HPLC) coupled to an electrochemical
87 detector (ED) revealed that levodopa is completely converted to a new metabolite, which was identified
88 by ^{1}H-NMR and LC-MS as 3-(3,4-dihydroxyphenyl) propionic acid, DHPPA (**Figure 1a,**
89 **Supplementary 1a, b**). Similarly, 5-HTP was converted into two new unknown peaks (**Figure 1a**),
90 albeit to a much lesser extent compared to levodopa. Only the first peak could be detected and assigned

91 by LC-MS as 5-hydroxyindole-3-lactic acid (5HILA) by its predicted exact mass (**Supplementary**
92 **Figure 1c**). To further investigate the involvement of the deaminase pathway in levodopa and 5-HTP
93 deamination, the enzyme responsible for the dehydratase reaction (encoded by the *fldC* gene) was
94 disrupted using the ClosTron mutagenesis system (**Supplementary Figure 2a**) (Heap et al., 2010). The
95 resulting strain *C. sporogenes Ll.LtrB-ery*^{R}*ΩfldC* (CS$^{\Omega fldC}$) was incubated with levodopa, revealing that
96 levodopa was no longer deaminated to DHPPA but to its intermediate product 3-(3,4-
97 dihydroxyphenyl)lactic acid (DHPLA) (**Figure 1b**). Tryptophan and tyrosine are converted to their
98 intermediates ILA (indole-3-lactic acid) and 4HPLA (3-(4-hydroxyphenyl)lactic acid), respectively.
99 Only a slight production of 4HPPA (from tyrosine) is observed after 48 h, potentially because of the
100 substitution of FldABC by the similar HadABC proteins from the *had*-operon in *C. sporogenes* (Dickert
101 et al., 2002; Dodd et al., 2017). HPLC-ED analysis of the 5HILA production from 5-HTP by the *fldC*
102 mutant was hampered by the production of coeluting 3-(4-hydroxyphenyl) lactic acid (4HPLA), a
103 metabolic intermediate deamination product produced from tyrosine. However, the analysis revealed
104 that the second unknown peak produced from 5-HTP was no longer produced by CS$^{\Omega fldC}$ (**Figure 1b**),
105 demonstrating that 5-HTP conversion is affected and suggesting that the unknown product is 5-
106 hydroxyindole-3-propionic acid (5HIPA). Overall, these results show that the deamination pathway
107 from *C. sporogenes* is not only involved in the deamination of PAAAs but also is important in the
108 deamination of NPAAAs like 5HTP and levodopa.

109 **Identification of the aromatic aminotransferase responsible for initiation of the deamination**
110 **pathway**

111 The aromatic aminotransferase responsible for the transamination of levodopa and the other (N)PAAAs,
112 is crucial for the initiation of the reductive deamination pathway. However, the gene encoding this
113 transaminase remains unidentified. To further investigate this critical step in the pathway, all nine class
114 I/II aminotransferases present encoded by *C. sporogenes* were cloned, purified and screened for their
115 activity on levodopa and the other (N)PAAAs, revealing a single aminotransferase (EDU38870 encoded
116 by CLOSPO_01732) to be involved in their transamination (**Figure 2a**). To verify whether other
117 aminotransferases could substitute for the identified aminotransferase *in vivo*, CLOSPO_01732 was

118 disrupted (resulting in $CS^{\Omega CLOSPO_01732}$ (**Supplementary Figure 2a**)) and a targeted metabolomic
119 analysis of all the (N)PAAA metabolites was performed using HPLC-ED (except metabolites from
120 phenylalanine, which were quantified using HPLC-UV). Comparing the metabolic profiles from wild
121 type *C. sporogenes* (CS^{WT}), $CS^{\Omega fldC}$ and $CS^{\Omega CLOSPO_01732}$ demonstrated that none of the other tested
122 aminotransferases could take over this transaminase reaction effectively, except for the substrate
123 phenylalanine (**Figure 2b and Supplementary Table 1**). Disrupting CLOSPO_01732 reduced the
124 production of phenyl-3-propionic acid (PPA), 3-(4-hydroxyphenyl)propionic acid (4HPPA), IPA, and
125 DHPPA by 16.4%, 79.0%, 97.2%, 97.7% respectively compared to CS^{WT} within 24 h. Presumably, the
126 transamination of phenylalanine is substituted by EDU37030 as this aminotransferase also showed
127 significant phenylalanine-converting activity *in vitro* (**Figure 2a**). Interestingly, $CS^{\Omega CLOSPO_01732}$
128 produces significantly higher amounts of tryptamine (4 to 6-fold increase at 24 and 48 h, respectively)
129 compared to CS^{WT} ($p \leq 0.0001$), reflecting a reduced competition for the same substrate by different
130 enzymes. Analogous to tryptamine, $CS^{\Omega CLOSPO_01732}$ produced significantly more serotonin compared to
131 CS^{WT} at 48 h ($p < 0.0001$) when incubated with 5-HTP (**Figure 2b**, **Supplementary Figure 2c,**
132 **Supplementary Table 1**), though to a much lesser extent compared to tryptamine. Notably, disrupting
133 *fldC* or *CLOSPO_01732* resulted in only a minor reduction of the exponential growth rate in rich
134 medium (T_D=55.1±1.2 min and 64.1±1.1 min respectively compared to wild type 44.3±1.2 min)
135 (**Supplementary Figure 2b**), which does not affect the observed production of the measured
136 metabolites after 24 or 48 h.

137 **DHPPA elicits an inhibitory effect on the acetylcholine-induced ileal muscle contractions**

138 Because levodopa, compared to 5-HTP, is efficiently deaminated to DHPPA within 24 h by *C.*
139 *sporogenes,* we further focused on the deamination product of this drug. DHPPA is a phenolic acid (a
140 molecule in the class polyphenols) and recent findings demonstrated an association between bacterial-
141 derived polyphenol metabolites and gut-transit times in humans (Roager et al., 2016). In order to test
142 whether DHPPA could also affect the gut motility, we suspended ileal rings of wild type C57BL/6 mice
143 in an *ex-vivo* organ bath system. Our initial results indicated that DHPPA displayed an inhibiting effect
144 on ileum contractility, therefore we tested whether DHPPA might have an inhibiting effect on

145 acetylcholine induced contractility in the ileum. Acetylcholine, produced from the excitatory circular
146 muscle motor neurons, is the neurotransmitter directly acting on gut smooth muscle contractility (Costa
147 et al., 2000). After 5 min, induction of an acetylcholinergic twitch by adding 50 µM of acetylcholine,
148 DHPPA (100 µM) specifically inhibited the amplitude (measured as the standard deviation of the mean),
149 but not the frequency of the acetylcholinergic twitch after 15 min (**Figure 3a,b**). When incubation times
150 were binned in intervals of 5 min a significant decrease (up to 60% reduction) in the acetylcholinergic
151 twitch by DHPPA was observed at the 10-15- and 15-20-min interval (**Figure 3c**), showing that DHPPA
152 can inhibit the acetylcholinergic twitch of mouse ileum *ex vivo*.

153 **Higher DHPPA concentrations in fecal samples from PD patients compared to healthy controls**

154 Although levodopa is mainly absorbed in the proximal small intestine, a significant amount reaches the
155 distal part of the intestinal tract (Morgan, 1971), and these levels increase with age (Iwamoto et al.,
156 1987). Thus, we hypothesized that PD patients have considerable amounts of DHPPA in their distal GI-
157 tract if *C. sporogenes* or other bacteria with such a pathway (*Peptostreptococcus anaerobius* or
158 *Clostridium cadaveris* (Dodd et al., 2017)) are present. To this end, we performed a catechol extraction
159 targeted for the quantification of DHPPA on 10 PD patients and 10 age-matched healthy controls (HC).
160 DHPPA concentrations were significantly higher in PD patient's fecal samples compared to HC (**Figure**
161 **4**) and results were confirmed by LC-MS (**Supplementary Table 2**). Overall the significantly higher
162 levels of DHPPA detected in the fecal samples of PD patients compared to HC indicate active bacterial
163 metabolism of residual levodopa.

164 **Active levodopa deamination pathway in fecal-suspensions**

165 The higher amounts of DHPPA observed in the fecal samples of PD patients are highly likely to result
166 from levodopa metabolized by the anaerobic bacteria deaminating levodopa through FldBC. In order to
167 test this hypothesis, 10% (w/v) fecal-suspensions from PD and HC were incubated anaerobically with
168 DHPLA, the product of levodopa before dehydration by bacterial FldBC proteins (**Figure 1**). Samples
169 were collected at 0, 20, and 45 h and were analyzed by HPLC-ED. After 20 h, DHPPA was clearly
170 detected in samples from PD7 and HC13 (**Figure 5a**), and was further converted to the downstream

171 dehydroxylated metabolite of DHPPA, 3-(3-hydroxyphenyl) propionic acid (3HPPA) over time (**Figure**
172 **5a**). For other samples, DHPPA detection was hampered by interference of other unknown peaks present
173 in the sample. Therefore the same samples were re-injected using a lower voltage on the ED (0.8 V)
174 making the detection more selective for more readily oxidizable compounds (Nagatsu and Kojima,
175 1988) such as DHPPA, but making 3HPPA invisible for detection. Using the lower voltage resulted in
176 the detection of DHPPA in 35% of all samples (**Supplementary Figure 3)**. Because DHPPA is further
177 converted to 3HPPA *in vitro* (**Supplementary Figure 3**), we quantified the production of 3HPPA in the
178 fecal incubations as measure for deamination. In 60% and 40% of PD and HC respectively, 3HPPA
179 production was observed within 45 h (**Figure 5b, Supplementary Figure 3**).

180 *Eggerthella lenta* has been shown to be able to perform *p*-dehydroxylation of the catecholic B-ring from
181 (+)-catechin and (-)-epicatechin (Jin and Hattori, 2012), a moiety resembling DHPPA. To test whether
182 *E. lenta* could produce 3HPPA from the dihydroxylation of DHPPA, *E. lenta* DSM 2243 was grown at
183 various concentrations of arginine, which was previously shown to improve growth densities (Haiser et
184 al., 2013). At low arginine concentrations, *E. lenta* DSM2243 was capable of dehydroxylation of
185 DHPPA to 3HPPA, which was inhibited at higher arginine concentrations (**Figure 5c**).

186 Taken together, the results show that DHPPA can be produced by the microbiota via anaerobic
187 deamination of levodopa. Moreover, our findings indicate that 3HPPA originates from DHPPA via
188 dehydroxylation potentially by *Eggerthella lenta* and that the aromatic deamination pathway, as
189 measured by the production of 3HPPA, is active in at least 50% of the PD and HC samples.

190 **Discussion and Conclusion**

191 Identifying bacterial pathways and elucidating their potential impact on bacterial drug metabolism is
192 crucial in order, not only, to maximize medication efficacy, but also to recognize and eventually prevent
193 potential side effects that might affect the host's physiology on an individual basis (Haiser et al., 2013;
194 van Kessel et al., 2019; Niehues and Hensel, 2009; Zimmermann et al., 2019). Here we disclosed the
195 reductive anaerobic deamination pathway in *C. sporogenes* by identifying its initiating enzyme, the
196 aromatic aminotransferase, and expanded the pathway's relevance by demonstrating its capacity to

197 convert two clinically important NPAAAs, levodopa and 5-HTP. We showed that *C. sporogenes* is able
198 to completely deaminate levodopa to DHPPA and to a much lesser extent 5-HTP (**Figures 1, 2**).
199 Disrupting the bacterial transaminase encoding gene abolished the production of deaminated products,
200 and increased the production of neuromodulators such as tryptamine and, to much lesser extent,
201 serotonin (**Figure 2, Supplementary Figure 2c and Supplementary Table 1**). Tryptamine is a natural
202 product produced by *C. sporogenes* that has been proposed to modulate gut transit time (Williams et al.,
203 2014). The application of engineered gut bacteria as a therapeutic strategy to modulate GI-motility or
204 host physiology has been also proposed recently in two proof-of-concept studies; by heterologous
205 expression of *Ruminococcus gnavus* tryptophan decarboxylase in *Bacteroides thetaiotamicron*
206 (Bhattarai et al., 2018) and by modification of the metabolic output of bioactive compounds in an
207 engineered *fldC* deficient *C. sporogenes* strain (Dodd et al., 2017). However, translation of these studies
208 into applications is hindered by restrictions on the application of genetically engineered microorganisms
209 (GMOs) *per se*, and the complexity of introducing these GMOs into an existing gut microbiota
210 ecosystem. Selective therapeutic blockage of the aminotransferase identified in this study may provide
211 an attractive alternative solution to modify gut microbiota metabolism.

212 PD patients encounter increased gut transit time; thus, an additional inhibition of acetylcholine-induced
213 contraction could result in further slowing down of gut transit rates. The inhibitory effect of DHPPA on
214 the acetylcholine-induced ileal muscle contractions (**Figure 3**), higher DHPPA levels in fecal samples
215 of PD patients compared to HC (**Figure 4**), and an active deamination pathway of levodopa during fecal-
216 incubations of PD patients (**Figure 5**) demonstrate active deamination of levodopa in the distal GI-tract
217 of PD patients and suggest potential side effects of this bacterial by-product of the unabsorbed residue
218 of the medication. DHPPA shares similarity with dopamine structure except of the terminal amine group,
219 which is substituted by a carboxyl group in DHPPA. Dopamine, and dopamine agonists, have been
220 shown to inhibit methacholine (analog of acetylcholine) induced contraction, which is not mediated via
221 dopamine receptors, in guinea pig jejunum in similar concentration ranges to DHPPA (EC_{50} relaxation
222 by dopamine ~290 μM) (Lucchelli et al., 1990), indicating that DHPPA might act on a similar receptor.
223 Although further research is needed to unravel the underlying mechanism, these results indicate that

224 DHPPA inhibits the acetylcholine-induced muscle contractions in the ileum with implications on
225 intestinal motility, often observed in PD patients. Overall, the present study highlights the urgency to
226 unravel potential effects of gut bacterial processing of (unabsorbed residues of) medication, such as
227 levodopa.

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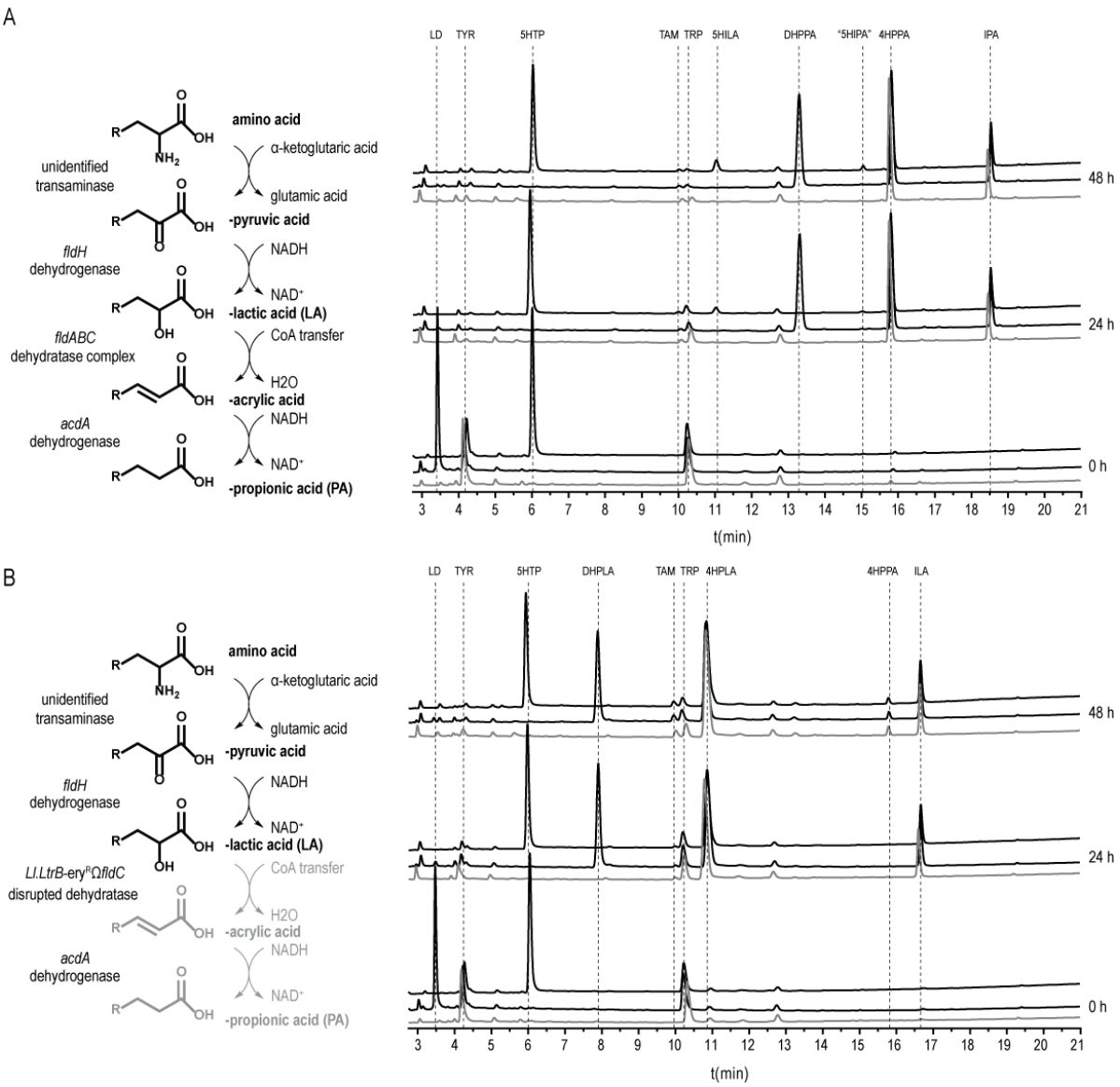

Figure 1. Levodopa is deaminated by *Clostridium sporogenes*. (A) Full reductive deamination pathway of *C. sporogenes* is depicted next to the HPLC-ED curves from supernatant of a *C. sporogenes* batch culture conversion of levodopa (3-(3,4-dihydroxyphenyl)alanine) or 5HTP (5-hydroxy-L-tryptophan) overtime. At the beginning of growth (timepoint 0 h) 100 µM of levodopa (middle black line) or 5HTP (upper black line) is added to the culture medium, the grey line depicts the control samples. In 24 h, levodopa is completely converted to DHPPA (3-(3,4-dihydroxyphenyl)propionic acid), the deaminated product of levodopa. 5HTP is only converted to a minor extent to 5HILA (5-hydroxyindole-3-lactic acid), as determined by LC-MS and potentially to 5HIPA (5-hydroxyindole-3-propionic acid), as this peak was absent in $CS^{\Omega fldC}$ incubations. Other aromatic amino acids from the medium, tryptophan and tyrosine (which are detectable with ED), are converted to the deaminated

240 products IPA (indole-3-propionic acid) and 4HPPA (3-(4-hydroxyphenyl) propionic acid). (B)
241 Disrupted deamination pathway of *C. sporogenes* is depicted, where the dehydratase subunit *fldC* is
242 mutagenized. Analysis of the supernatant of $CS^{\Omega fdlC}$ shows that levodopa is not deaminated to DHPPA
243 but to its intermediate product DHPLA (3-(3,4-dihydroxyphenyl) lactic acid) within 24 h. Tryptophan
244 and tyrosine are converted to their intermediates ILA (indole-3-lactic acid) and 4HPLA (3-(4-
245 hydroxyphenyl) lactic acid), respectively.

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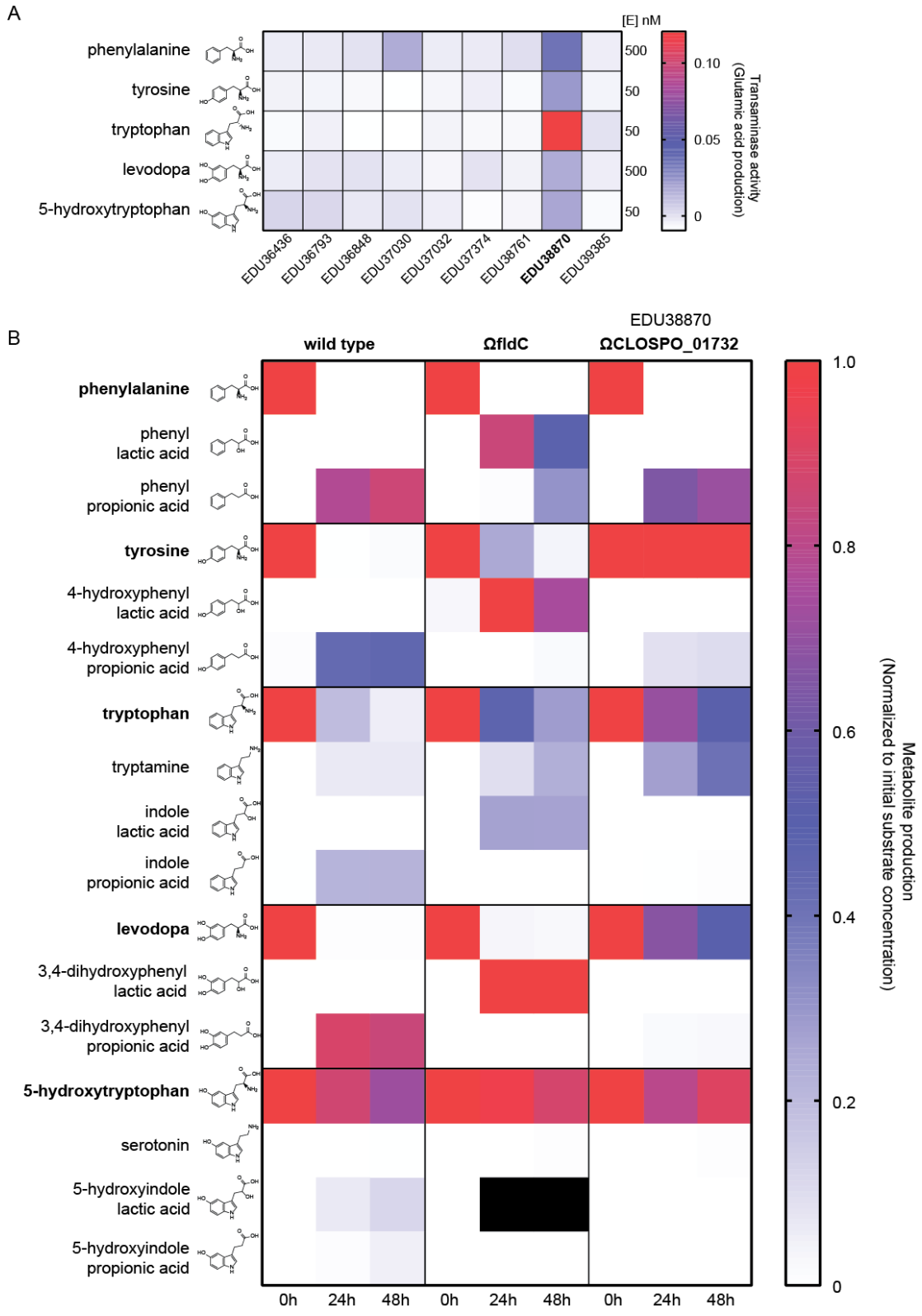

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248 **Figure 2. Identification of the aromatic amino transferase initiating the deamination pathway.** In
249 order to identify which aminotransferase is responsible for the initial transaminase reaction all class I/II
250 aminotransferase were cloned and purified to test the activity against (N)PAAAs. (A) Transaminase

251 activity (production of glutamate) for all substrates is depicted. EDU38870 (CLOSPO_01732) seemed
252 to be involved in all transaminase reactions. EDU37030 showed similar activity as EDU38870, for
253 phenylalanine. (B) Targeted metabolic quantification of deamination products from CS^{WT}, $CS^{\Omega fldC}$,
254 $CS^{\Omega CLOSPO_01732}$ reveals that EDU38870 is involved in the transamination of all for all tested (N)PAAAs.
255 All quantified deamination products are normalized to their initial substrate concentration and the data
256 represents 3 independent experiments. Black squares indicate that quantification was not possible
257 because of an interfering HPLC-ED peak. As no commercial standards are available for 5HILA and
258 5HIPA, the peaks were quantified assuming a similar ED-detector response as for 5HTP. Corresponding
259 values are given in Supplementary Table 2. Significance was tested when relevant (see p-values in text)
260 using 2-way ANOVA followed by a Tukey test.

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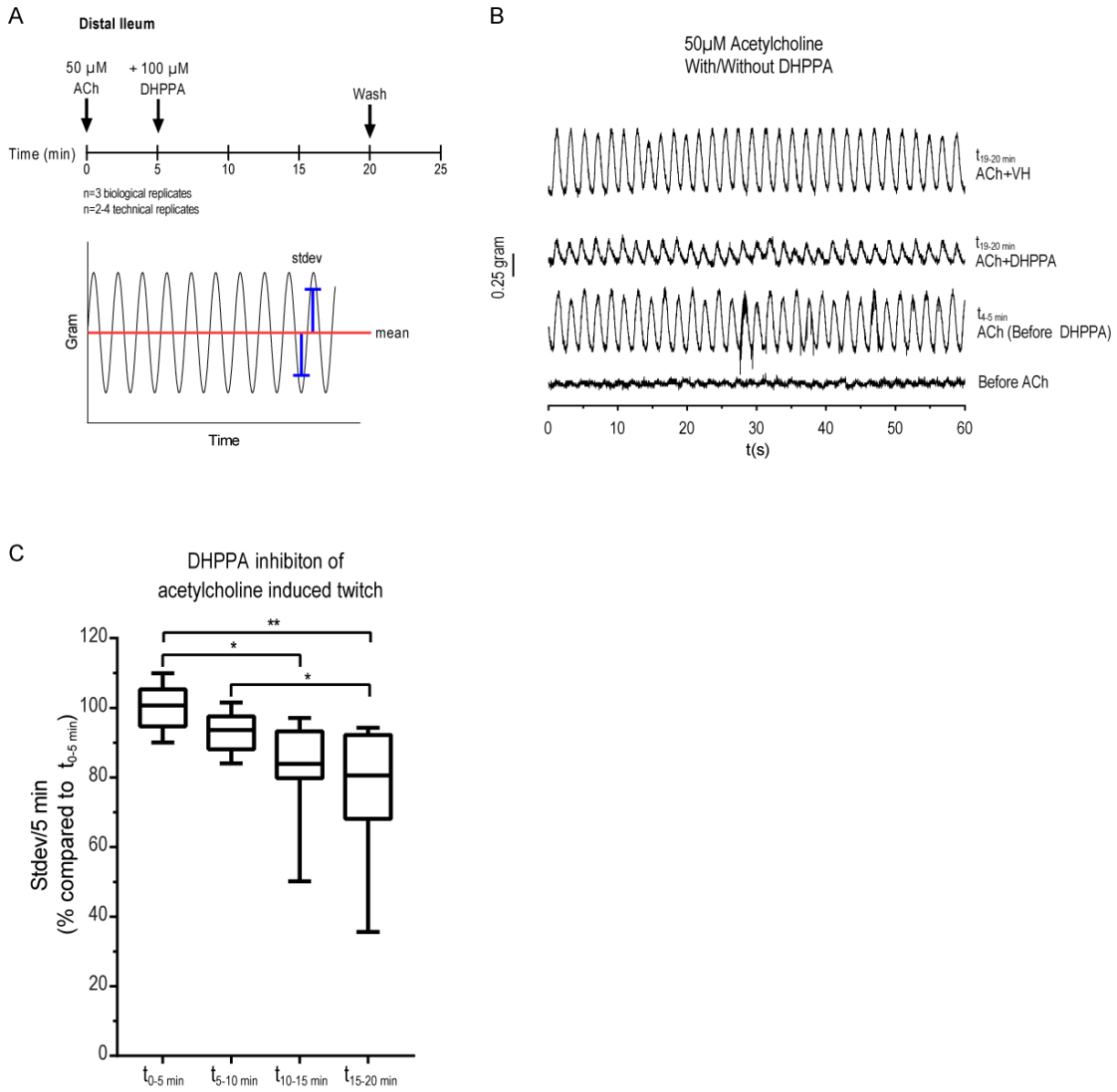

Figure 3. DHPPA inhibits the acetylcholine induced twitch from mouse ileum. (A) the experimental setup is depicted, where 5 min after adding 50 µM acetylcholine, 100 µM DHPPA is added. The panel below indicates how the amplitude is measured (the standard deviation of the mean). (B) a representative recording trace of 1 min before and after the addition of acetylcholine and DCHA or vehicle is shown. In (C) the inhibition of DHPPA on acetylcholine induced twitch binned in intervals of 5 minutes shows a decrease in contractibility over time (n=3 biological replicates and experiments were repeated 2-4 times per tissue). ACh, acetylcholine; VH, vehicle (0.05% ethanol). Significance was tested using 1-way-ANOVA followed by a Tukey's test (p-values: *<0.033 **<0.0021).

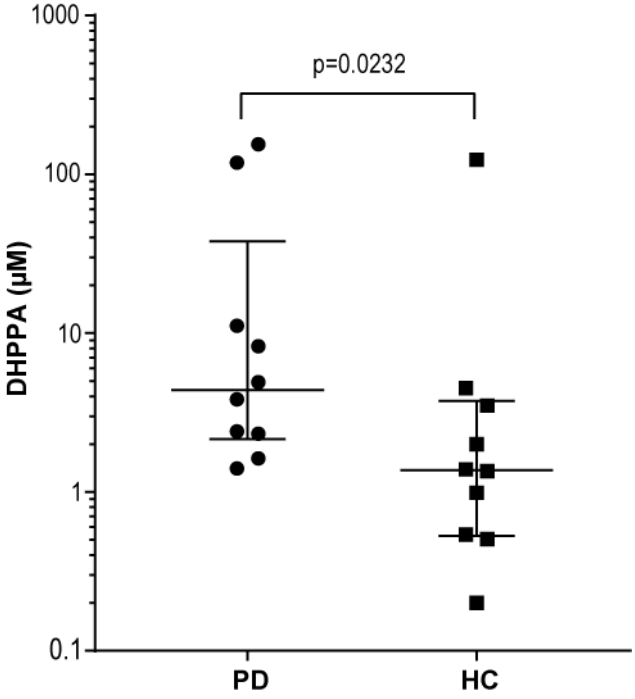

Figure 4. DHPPA concentrations are significantly higher in PD patients compared to healthy controls. DHPPA was extracted from stool samples of PD patients (n=10) and age-matched healthy controls (n=10) using activated alumina beads and concentrations were quantified using a standard curve of DHPPA on the HPLC-ED (DC amp, 0.8 V) with 3,4-dihydroxybenzylamine as internal standard. DHPPA concentrations are depicted on the logarithmic y-axis and individual levels are indicated and compared between PD patients (PD) and age-matched healthy controls (HC). The cross-header represents the median (PD, 4.36 µM; HC, 1.37 µM) and the interquartile range (PD, 2.15-37.90 µM; HC, 0.53-3.75 µM).

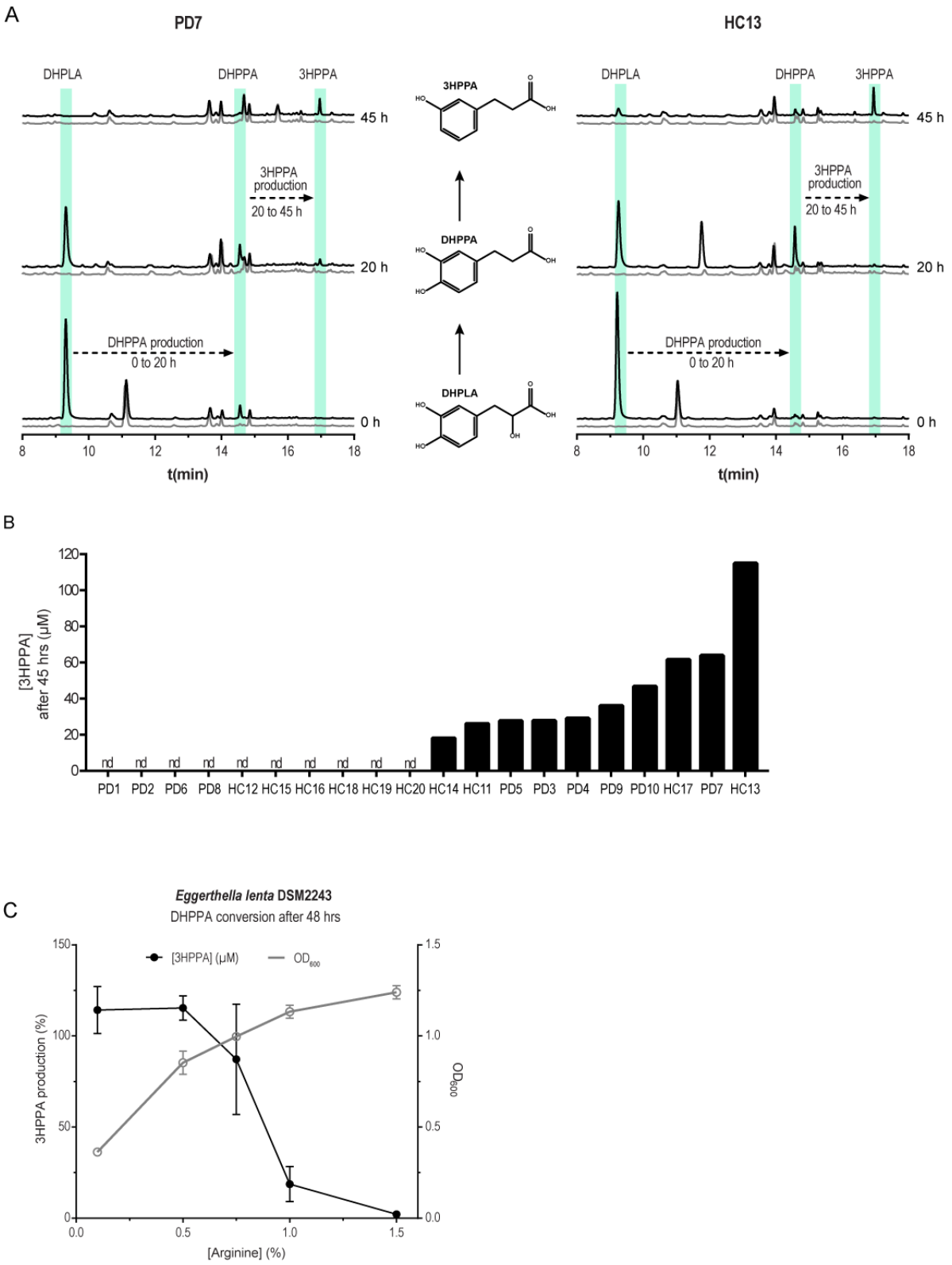

Figure 5. Active levodopa deamination pathway in fecal-suspensions. (A) Fecal-suspension incubation of PD7 and HC13 are shown, where DHPPA is produced from DHPLA after 20 h and is further metabolized to 3HPPA after 45 h of incubation. Green bars indicate the retention time of the

286 standards indicated. (B) 3HPPA was quantified as measure for active deamination in the fecal-
287 suspensions. 3HPPA is produced in 60% of the PD patient's fecal-suspensions and in 40% of the HC's
288 fecal-suspensions. In (c) the dehydroxylation of DHPPA to 3HPPA by *Eggerthella lenta* DSM2243
289 dependent on the arginine concentration in the medium is shown. On the left y-axis 3HPPA production
290 is indicated, on the right y-axis the optical density (OD) at 600 nm is indicated, and on the x-axis the
291 increasing arginine concentration supplied to the medium before 48 h of incubation with 50 µM of
292 DHPPA.

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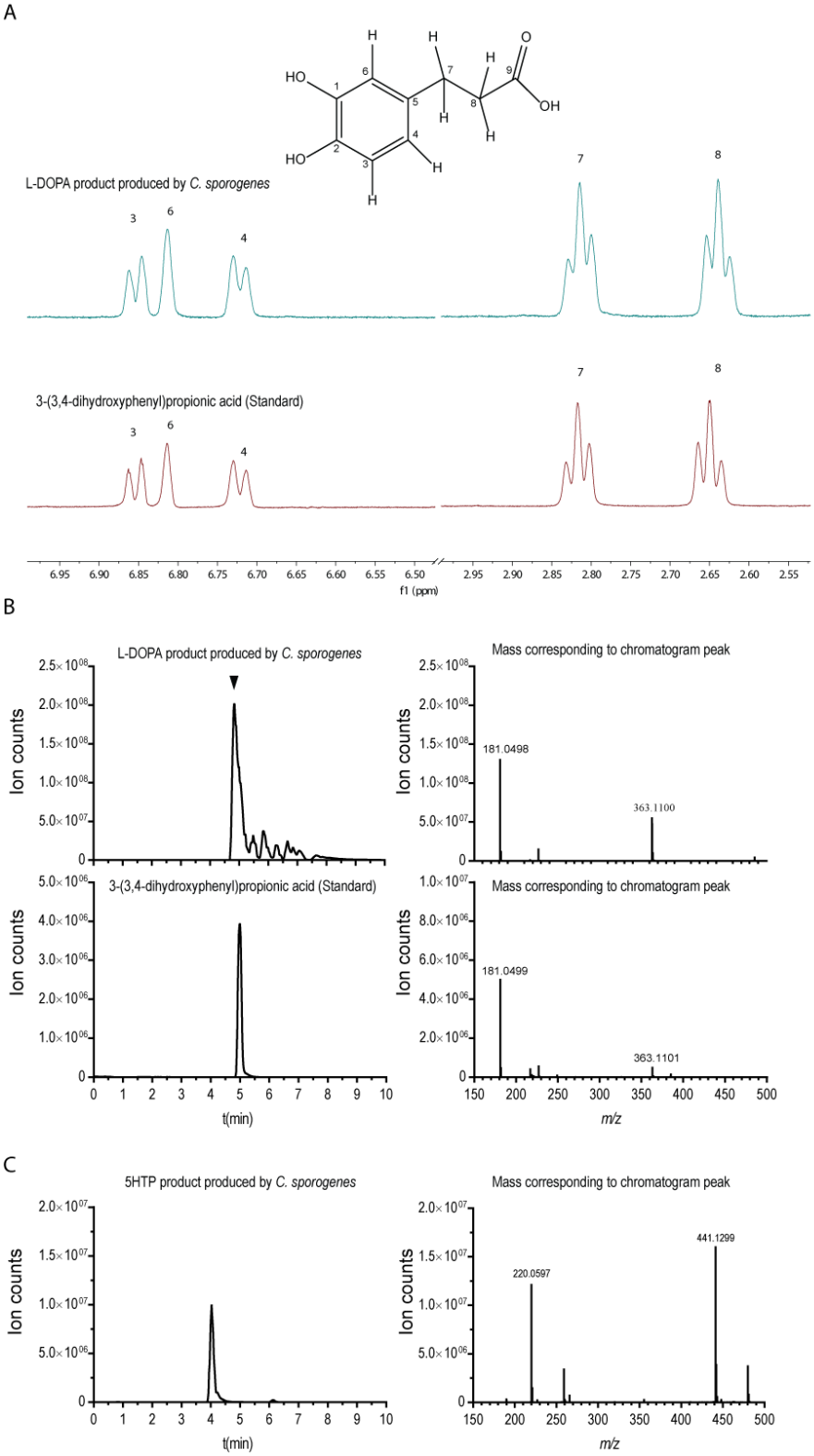

Supplementary Figure 1. NMR and MS confirmation of levodopa and 5HTP conversion. (A) 1D ^{1}H-NMR of the levodopa isolated product from *C. sporogenes* is compared with the commercially available standard of DHPPA. (B) the LC-ESI-MS in negative mode show the exact same mass and retention time as the commercially available standard of DHPPA. (C) LC-ESI-MS analysis shows the

299 mass of the first peak produced by CS^{WT} from 5-HTP isolated from the HPLC-ED corresponding to the

300 mass of 5HILA (predicted exact mass 221.069-[H^+]).

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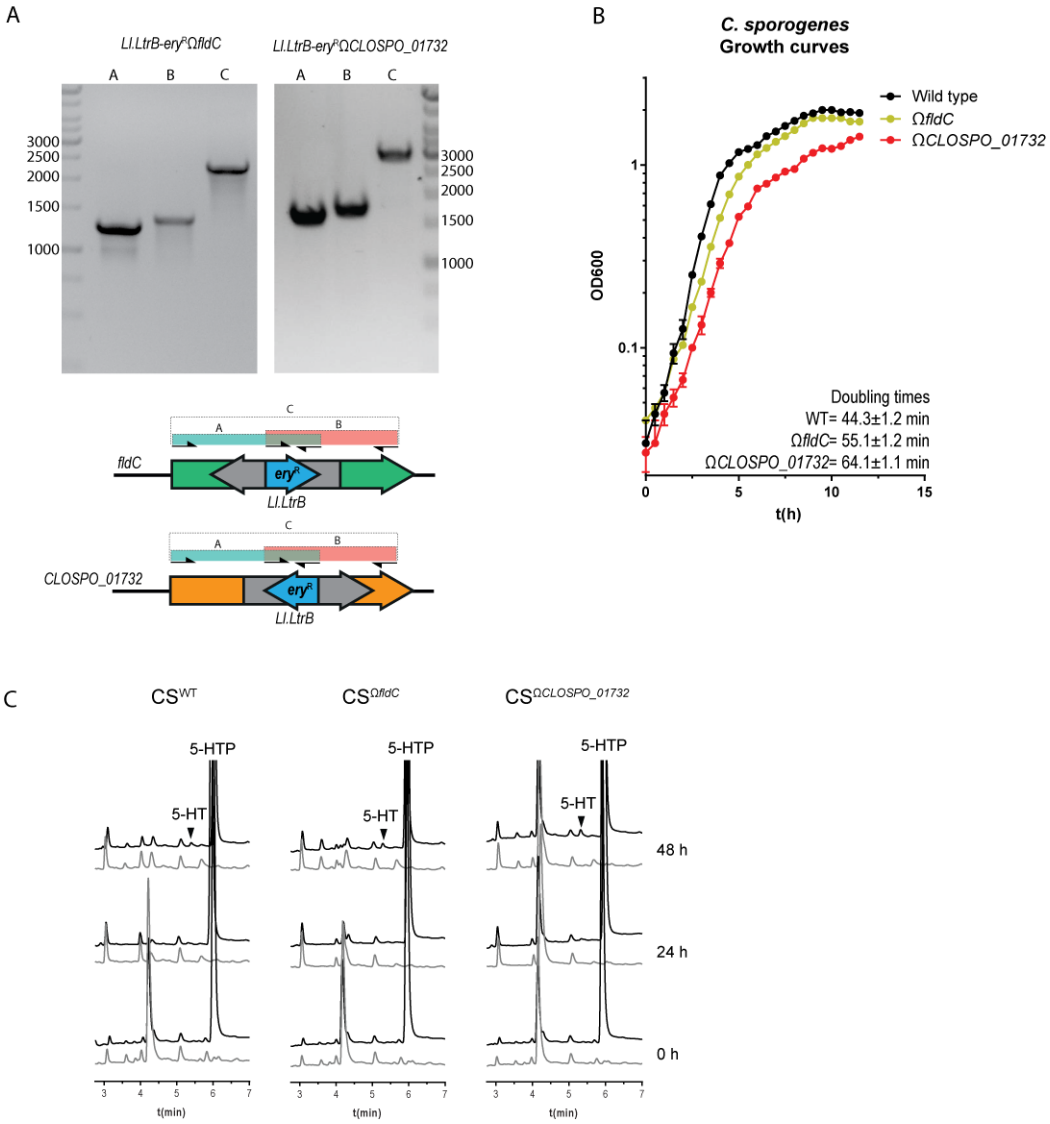

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303 **Supplementary Figure 2. Confirmation and growth curves of CS$^{\Omega fldC}$ and CS$^{\Omega CLOSPO_01732}$, and 5-**

304 **HT production.** (A) primers targeting the erythromycin cassette in *Ll.LtrB* intron and primers binding

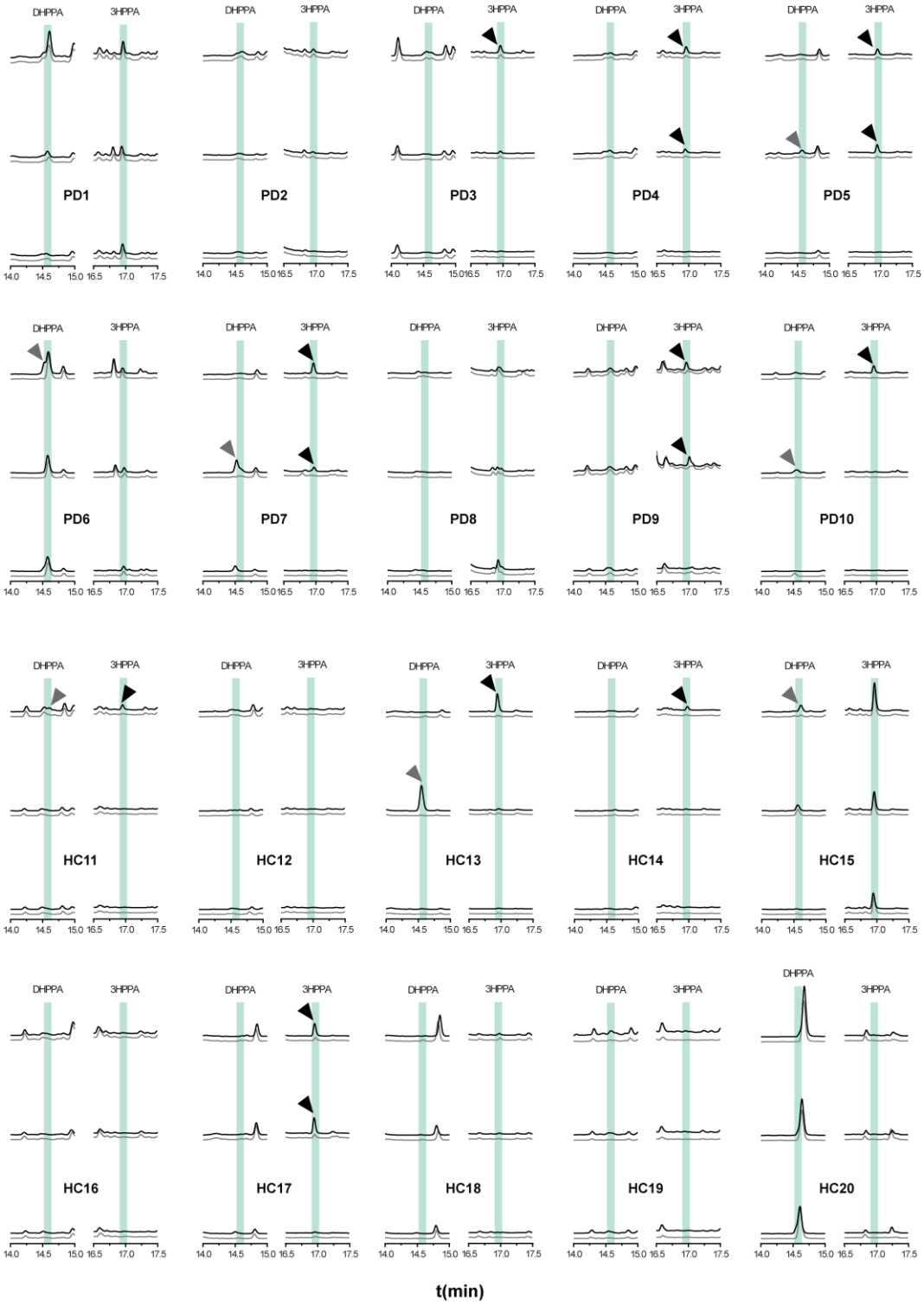
305 outside the cassette were used to confirm the disruption of the *fldC* and *CLOSPO_01732*. (B) Growth-

306 curves of CS^{WT}, CS$^{\Omega fldC}$ and CS$^{\Omega CLOSPO_01732}$ showing minor but significant increase in doubling time in

307 the first part of the growth curve, indicating that the TA/FldC is potentially important for a metabolic

308 reaction required for optimal growth. In (C) a minor production of 5-HT (serotonin) is observed in all

309 strains after 48 h.



310

311 **Supplementary Figure 3. Chromatograms of fecal-incubations from PD patients (n=10) and**
312 **healthy age-matched controls (n=10).** Green bars indicate the location of the standards DHPPA (3-
313 (3,4-dihydroxyphenyl)propionic acid) and 3HPPA (3-(3-hydroxyphenyl)propionic acid), black and grey
314 lines indicate DHPLA (3-(3,4-dihydroxyphenyl)lactic acid) and control samples, respectively. The
315 chromatograms from 14-15 min are from samples analyzed on DC amp = 0.8 V, making the detection

316 more selective for DHPPA, and chromatograms from 16.5-17.5 min are from the same samples analyzed
317 on DC amp = 1.0 V, making the detection of 3HPPA possible. In PD samples 5, 6, 7, 10 and HC samples
318 11, 13, 15 DHPPA production is observed in 20-45 h samples, indicated by the grey arrow. In PD
319 samples 3, 4, 5, 7, 9, 10 and HC samples 11, 13, 14, 17, 3HPPA production is observed in 20-45 h
320 samples, indicated by the black arrow. In samples PD 5, 7, 10 and HC 13, DHPPA is visible at 20 h, and
321 further converted to 3HPPA visible in the 45 h sample.

322

323 **Supplementary Table 1.** Values corresponding to Figure 2b

	WT			*ΩfldC*			ΩCLOSPO_01732		
	0 h	**24 h**	**48 h**	**0 h**	**24 h**	**48 h**	**0 h**	**24 h**	**48 h**
Phenylalanine	100.0 ± 17.7	n.d.	n.d.	100.0 ± 15.9	n.d.	n.d.	100.0 ± 4.6	n.d.	n.d.
PLA	n.d.	n.d.	n.d.	n.d.	84.7 ± 13.2	48.4 ± 9.1	n.d.	n.d.	n.d.
PPA	n.d.	77.6 ± 3.3	85.6 ± 0.7	n.d.	1.5 ± 0.3	30.7 ± 4.1	n.d.	64.9 ± 3.8	71.6 ± 3.1
Tyrosine	100.0 ± 23.0	0.4 ± 0.2	1.7 ± 1.0	100.0 ± 16.8	25.1 ± 12	4 ± 0.7	100.0 ± 18.1	110.9 ± 15.9	109.3 ± 18.2
4HPLA	n.d.	n.d.	n.d.	3.2 ± 0.7	106.9 ± 15.3	75.0 ± 39.8	n.d.	n.d.	n.d.
4HPPA	1.5 ± 0.3	43.7 ± 5.0	45.6 ± 4.9	n.d.	n.d.	1.9 ± 1.3	n.d.	9.2 ± 0.6	10.2 ± 0.4
Tryptophan	100.0 ± 21.4	19.6 ± 10.0	5.4 ± 4.1	100.0 ± 20.3	47.3 ± 15.3	28.3 ± 9.3	100.0 ± 16.6	70.7 ± 17.5	50.9 ± 13.6
Tryptamine	n.d.	6.7 ± 2.3	7.0 ± 1.9	n.d.	9.6 ± 2.4	23.5 ± 2.6	n.d.	27.8 ± 1.2	40.8 ± 4.4
ILA	n.d.	n.d.	n.d.	n.d.	27.2 ± 1.9	27.0 ± 2.9	n.d.	n.d.	n.d.
IPA	0.6 ± 0.2	21.7 ± 1.3	22.2 ± 1.8	n.d.	n.d.	n.d.	n.d.	0.6 ± 0	0.8 ± 0.1
Levodopa	100.0 ± 36.4	1.0 ± 0.5	0.9 ± 0.4	100.0 ± 32.7	3.4 ± 1.1	2.9 ± 0.9	100.0 ± 23.1	67.5 ± 10.4	50.3 ± 8.5
DHLA	n.d.	n.d.	n.d.	n.d.	172.1 ± 45.2	158.6 ± 38.8	n.d.	n.d.	n.d.
DHPPA	n.d.	89.2 ± 24.1	84.4 ± 23.1	n.d.	n.d.	n.d.	n.d.	2.1 ± 0.1	2.6 ± 0.6
5HTP	100.0 ± 16.2	86.7 ± 12.6	72.1 ± 12.0	100.0 ± 17.1	96.4 ± 13	88.3 ± 14.2	100.0 ± 15.4	79.7 ± 30.9	91.0 ± 14.8
Serotonin	0.04 ± 0.04	0.21 ± 0.03	0.47 ± 0.07	0.04 ± 0.04	0.15 ± 0.05	0.7 ± 0.11	0.06 ± 0.03	0.23 ± 0.06	1.07 ± 0.12
"5HILA"	n.d.	6.6 ± 0.4	12.1 ± 0.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
"5HIPA"	n.d.	1.3 ± 0.3	5.2 ± 1.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d., not detected; Deamination products are normalized to their initial substrate concentrations (100%) ± values indicate SEM (n=3).
PLA, 3-phenyllactic acid; PPA, 3-phenylpropionic acid; 4HPLA, 3-(4-hydroxyphenyl)lactic acid; 4HPPA, 3-(4-hydroxyphenyl)propionic acid; ILA, 3-indolelactic acid; IPA, 3-indolepropionic acid; DHLA 3-(3,4-dihydroxyphenyl)lactic acid; DHPPA, 3-(3,4-dihydroxyphenyl)propionic acid; 5HTP, 5-hydroxytryptophan; 5HILA, 5-hydroxyindole-3-lactic acid; 5IPA, 5-hydroxyindole-3-propionic acid;

324

325 **Supplementary Table 2. MS confirms that DHPPA is extracted from PD and HC samples using**

326 **alumina.**

Sample	M-H	ppm error	DHPPA quantification alumina extraction (µM)
DHPPA	181.0507	5.1	
P1	181.0509	7.5	118.2
P2	181.0504	4.7	4.9
P3	181.0504	4.7	1.6
P4	181.0505	5.3	3.8
P5	181.0503	4.2	2.3
P6	181.0509	7.3	154.3
P7	ND		1.4
P8	181.0506	5.8	8.2
P9	181.0505	5.3	11.1
P10	181.0504	4.7	2.4
HC11	181.0506	5.8	2.0
HC12	181.0504	4.7	1.4
HC13	ND		0.5
HC14	181.0506	5.8	1.0
HC15	ND		0.2
HC16	ND		3.5
HC17	181.508	6.9	1.4
HC18	ND		0.5
HC19	181.0507	5.1	4.5
HC20	181.0509	7.3	123.5

327

328 **Supplementary Table 3 Plasmids and primers used in this study**

Plasmid	**Description**		**Reference**
pET15b	His-Tag, amp^R		Novagen
pET28b	His-Tag, kan^R		Novagen
pSK023	pET15b-EDU36436		This study
pSK024	pET15b- EDU36793		This study
pSK025	pET15b-EDU36848		This study
pSK026	pET15b-EDU37030		This study
pSK027	pET28b-EDU37032		This study
pSK028	pET15b-EDU37374		This study
pSK029	pET15b-EDU38761		This study
pSK030	pET15b-EDU38870		This study
pSK031	pET15b-EDU39385		This study
Accession	**Locus Tag**	**Primers used for cloning (5'-3')**	
EDU36436	CLOSPO_02604	sk173 FW: GCTACGCATATGAAGTTATCTAAAAAAGCAGTAG sk174 RV: ATTATTCTCGAGCTTTCTAACATTTTATCCACCTC	
EDU36793	CLOSPO_02962	sk177 FW: GCGCGCCATATGAAAAATAAATTTTTAGCCTATAAG sk178 RV: AATAATCTCGAGCCTCTGAAGCCAAGAAATCTG	
EDU36848	CLOSPO_03017	sk179 FW: CGCGCGCATATGAAATATGATTTTGATGAAATC sk180 RV: AATAATCTCGAGGCATTTCATAAAAACCCTAGC	
EDU37030	CLOSPO_03199	sk181 FW: GGCCGCCATATGAAATTTTCAAAAAGAATATCTGACAT sk182 RV: ACGTACCTCGAGGGGTAAGTTCTGAAAATAAAGTA	
EDU37032	CLOSPO_03201	sk215 FW: CGCGCGCTAGCGTGTTATTTAATGACAAATTAAGAC sk217 RV: GCGCGCCTCGAGTTTATAATATTTATCTAAAACTTTACCTAATC	
EDU37374	CLOSPO_03543	sk185 FW: GCGCGCCATATGAAGTATAATTTTGACAAAGTAG sk186 RV: GTACACCTCGAGTCCCTCCCATAATTTCAC	
EDU38761	CLOSPO_01623	sk191 FW: GCAAGCCATATGTTGTTTAAAAAAGGTGGTATTTAT sk192 RV: AAGAATCTCGAGCTTCACTTTAAAGGGAATTTTC	
EDU38870	CLOSPO_01732	sk193 FW: GCCGGCCATATGATTTCAAATGAAATGCTTAATC sk194 RV: AGTATACTCGAGCAGTTAATTAGCGGTTGTCC	
EDU39385	CLOSPO_00463	sk197 FW: GCGCATCATATGGATTATATGAAAACTCAAGAAG sk198 RV: AGTAATCTCGAGTCTCAACCTTTAAAGAATGTTAAG	
*restrictions sites are underlined			

329

330

Material and Methods

Growth and incubation of *Clostridium sporogenes* and *Eggerthella lenta*

Clostridium sporogenes ATCC15579 was grown in enriched beef broth (EBB) with 2 g/L glucose (van Kessel et al., 2019) and 0.1% Tween 80 (EBB/T) anaerobically (10% H_2, 10% CO_2, 80% N_2) in a Don Whitley Scientific DG250 Workstation (LA Biosystems, Waalwijk, The Netherlands) at 37 °C. *Eggerthella lenta* DSM2243 was grown in modified DSMZ medium 78 (DSMZ 78: Beef extract, 10.0 g/L; Casitone, 30.0 g/L; Yeast extract, 5.0 g/L; K_2HPO_4, 5.0 g/L; Tween 80, 0.1%; Menadione (Vitamin K3), 1 µg/ml; Cysteine, 0.5 g/L; Hemin, 5 mg/L; L-Arginine0.1-1.5%) anaerobically (1.5% H_2, 5% CO_2, balance with N_2) in a Coy Laboratory Anaerobic Chamber (neoLab Migge GmbH, Heidelberg, Germany) at 37 °C in a tube shaker at 500 RPM. Upon use, bacteria were inoculated from -80 °C stored glycerol stocks in the appropriate media and grown for 18-24 h for *C. sporogenes* and 24-40 h for *E. lenta*. Overnight turbid cultures were then diluted 1/50 in an appropriate volume EBB/T or CMM for further experiments with 100 µM levodopa (D9628, Sigma), 5-hydroxytrytophan (H9772, Sigma), 50 µM 3-(3,4-dihydroxyphenyl)propionic acid (102601, Sigma) or H_2O as control.

Protein production and purification

Transaminase-encoding genes from *C. sporogenes* (**Supplementary Table 3**) were amplified using Phusion High-fidelity DNA polymerase and primers listed in **Supplementary Table 3**. All amplified genes were cloned in pET15b, except for EDU37032 which was cloned in pET28b (**Supplementary Table 3)**. Plasmids were maintained in *E. coli* DH5α and verified by Sanger sequencing before transformation to *E. coli* BL21 (DE3). Overnight cultures were diluted 1:50 in fresh LB medium with the appropriate antibiotic and grown to OD600 = 0.7–0.8 shaking at 37 °C. Protein translation was induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG, 11411446001, Roche Diagnostics) and cultures were incubated overnight at 18 °C. The cells were washed with 1/5th of the volume in 1× ice-cold PBS and stored at −80 °C or directly used for protein isolation. Cell pellets were thawed on ice and resuspended in 1/50th of buffer A (300 mM NaCl; 10 mM imidazole; 50 mM KPO4, pH 8.0) containing 0.2 mg/mL lysozyme (105281, Merck) and 2 µg/mL DNAse (11284932001, Roche

357 Diagnostics), and incubated for at least 10 min on ice before sonication (10 cycles of 15 s with 30 s
358 cooling at 8 microns amplitude) using Soniprep-150 plus (Beun de Ronde, Abcoude, The Netherlands).
359 Cell debris was removed by centrifugation at 20,000 × g for 20 min at 4 °C. The 6 × his-tagged proteins
360 were purified using a nickel-nitrilotriacetic acid (Ni-NTA) agarose matrix (30250, Qiagen). Cell-free
361 extracts were loaded on 0.5 ml Ni-NTA matrixes and incubated on a roller shaker for 2 h at 4 °C. The
362 Ni-NTA matrix was washed three times with 1.5 mL buffer B (300 mM NaCl; 20 mM imidazole; 50
363 mM KPO4, pH 8.0) before elution with buffer C (300 mM NaCl; 250 mM imidazole; 50 mM KPO4,
364 pH 8.0). Imidazole was removed from purified protein fractions using Amicon Ultra centrifugal filters
365 (UFC505024, Merck) and washed three times and reconstituted in buffer D (50 mM Tris-HCl; 30 0mM
366 NaCl; pH 7.5). Protein concentrations were measured spectrophotometrically (Nanodrop 2000, Isogen,
367 De Meern, The Netherlands) using the predicted extinction coefficient and molecular weight from
368 ExPASy ProtParam tool (www.web.expasy.org/protparam/).

369 **Transaminase activity test**

370 Purified transaminases were incubated with 1 mM substrate, 2 mM α-ketoglutaric acid, and 0.1 mM
371 PLP (pyridoxal-5-phosphate, P9255, Sigma, The Netherlands) in buffer D with an enzyme concentration
372 of 50 nM for tyrosine, tryptophan, or 5-HTP as substrate and an enzyme concentration of 500 nM for
373 phenylalanine and levodopa as substrate. Enzyme reactions were incubated for 0.5 h at 37 °C, the
374 reactions were stopped with 0.7% (v/v) perchloric acid (1:1). Transaminase activity was tested using an
375 L-glutamic acid detection kit (K-GLUT, Megazyme Inc., Wicklow, Ireland), according to the
376 manufacture's microplate assay procedure with some modifications. The supplied buffer was substituted
377 for buffer D (described above, to prevent oxidation of the substrates/products). A reaction mix was
378 prepared mixing 50 µL buffer D; 10 µL quenched sample reaction mixture, 20 µL
379 NAD^+/iodonitrotetrazolium chloride solution, 5 µL diaphorase solution, 5 µL glutamate dehydrogenase
380 (GIDH) solution and reconstituted to a final volume of 290 µL with H2O. Absorbance at 492 nm was
381 measured after 10 minutes of incubation using a microplate reader (Synergy HTX spectrophotometer,
382 BioTek, BioSPX, The Netherlands) and background was subtracted from initial read before addition of
383 GIDH solution.

384 **Targeted mutagenesis**

385 Gene disruptions in *Clostridum sporogenes* were performed using the ClosTron system (Heap et al.,
386 2007, 2009). This system facilitates targeted mutagenesis using the group-II *Ll.LtrB* intron of
387 *Lactococcus lactis*. Introns targeting *fldC* (CLOSPO_311) or CLOSPO_1732 (encoding for the
388 transaminase) were designed using the ClosTron intron design tool (http://www.clostron.com) and were
389 ordered in pMTL007C-E2 from ATUM (Newark, California, United States) resulting in pMTL007C-
390 E2_Cs-fldC-561a and pMTL007C-E2_Cs-CLOSPO_1732-493s respectively. Plasmids were
391 transferred to *C. sporogenes* by conjugation as described before (Heap et al., 2009) using *E. coli* CA434
392 (*E. coli* HB101 (Bio-Rad Laboratories, The Netherlands) harboring the broad host IncPß$^{+}$ conjugational
393 plasmid pRK24 (Williams et al., 1990) as donor strain. *E. coli* CA434 harboring pMTL007C-E2_Cs-
394 fldC-561a or pMTL007C-E2_Cs-CLOSPO_1732-493s was grown in Luria Broth (LB) with 10 µg/mL
395 tetracycline and 25 µg/mL chloramphenicol (to select for pRK24 and PMTL007C-E2 respectively). Cell
396 suspensions of 1 mL of overnight culture were washed once with PBS and the cell pellet was
397 resuspended in 200 µL of *C. sporogenes* overnight cell suspension. The bacterial-mixture was spotted
398 (in drops of 10 µL) on trypticase soy agar (TSA) plates and incubated for 24 h anaerobically at 37 °C.
399 Sequentially, 1 mL of PBS was added to the spotted plates and the donor-recipient mix was scraped of
400 the plate, sequentially the scraped-off suspension was distributed over TSA plates containing 50 µg/mL
401 neomycin (to prevent growth of *E. coli*) and 15 µg/mL chloramphenicol to select for *C. sporogenes*
402 conjugants. Chloramphenicol resistant colonies of *C. sporogenes* were re-streaked on TSA plates
403 containing 50 µg/mL neomycin and 2.5 µg/mL erythromycin (to select for intron insertion) for several
404 times. To makes sure the plasmids was integrated, colonies were checked and selected for their
405 sensitivity towards chloramphenicol and the genomic DNA was verified using PCR (**Supplementary**
406 **Figure 2a**)

407 **Fecal samples from patients with Parkinson's disease and age-matched healthy controls**

408 Fecal samples from patients diagnosed with PD (n = 10) and age-matched healthy controls (n = 10) were
409 acquired from the Movement Disorder Center at Rush University Medical Center, Chicago, Illinois,
410 USA published previously (Keshavarzian et al., 2015). All study subjects consented to the use of their

411 samples for research. PD was diagnosed according to the UK Brain Bank Criteria as previously
412 described (Keshavarzian et al., 2015). Study subjects were provided with the supplies and instructions
413 for home feces collection using the BD Gaspak EZ Anaerobe Gas Generating Pouch System with
414 Indicator (Ref 260683; Becton, Dickinson and Company, Sparks, MD) in order to minimize the
415 exposure of the feces to high oxygen ambient atmosphere, which may alter the microbiota. Subjects
416 were asked to have a bowel movement within 24 h of their study visit. Subjects kept the sealed anaerobic
417 fecal bag in a cold environment, before bringing the anaerobic fecal bag to the hospital. Fecal samples
418 were then immediately stored at -80°C until analysis.
419

420 **Fecal metabolite incubations from PD patients and HC subjects**

421 Stool samples were suspended 1:1 (w/v) in EBB/T and incubated anaerobically (10% H_2, 10% CO_2,
422 80% N_2) in a Don Whitley Scientific DG250 Workstation (LA Biosystems, Waalwijk, The Netherlands)
423 at 37 °C with 100 µM Sodium 3-(3,4-dihydroxyphenyl)-DL-lactate (39363,Sigma). Samples were taken
424 at 0, 20, and 45 h and analyzed on HPLC-ED as described below.
425

426 **HPLC-ED/UV analysis and sample preparation**

427 For bacterial cell suspensions, 1 mL of methanol was added to 0.25 mL of cell suspension and stored at
428 -20 °C until further use. For fecal metabolite incubations, 300 µl of methanol was added to 75 µL of
429 fecal suspension and stored at -20°C until further use. Metabolites from stool samples were extracted by
430 suspending the stool 1:1 (w/v) in water, followed by homogenization by vigorously vortexing while
431 keeping samples as cold as possible. Homogenized suspensions were centrifuged at 3500 × *g* for 20 min
432 at 4°C and sequentially 1.6 mL of methanol was added to 0.4 mL of supernatant. From bacterial, fecal
433 incubation or stool samples cells and protein precipitates were removed by centrifugation at 20000 × *g*
434 for 10 min at 4°C. Supernatant was transferred to a new tube and the methanol fraction was evaporated
435 in a Savant speed-vacuum dryer (SPD131, Fisher Scientific, Landsmeer, The Netherlands) at 60°C for
436 1.5-2 h. The aqueous fraction was reconstituted with 0.7% $HClO_4$ to the appropriate volume. Samples
437 were filtered and injected into the HPLC-ED system (Alliance Separations Module 2695, Waters
438 Chromatography B.V, Etten-Leur, The Netherlands; Dionex ED40 electrochemical detector, Dionex,

439 Sunnyvale, USA, with a glassy carbon working electrode (DC amperometry at 0.8 or 1.0 V, with
440 Ag/AgCl as reference electrode)). Samples were analyzed on a C18 column (Kinetex 5µM, C18 100 Å,
441 250 × 4.6 mm, Phenomenex, Utrecht, The Netherlands) using a gradient of water/methanol with 0.1%
442 formic acid (0-10 min, 95−80% H_2O; 10-20 min, 80-5% H_2O; 20-23 min 5% H_2O; 23-31 min 95%
443 H_2O). For the detection of the *C. sporogenes* metabolites and for peak isolation another HPLC-ED
444 system was used (Jasco AS2059 plus autosampler, Jasco Benelux, Utrecht, The Netherlands; Knauer K-
445 1001 pump, Separations, H. I. Ambacht, The Netherlands) with the same detector (ED40) and the same
446 gradient as described above. Phenylalanine metabolites were detected by injecting the same samples in
447 an HPLC-UV system (Alliance Separations Module 2695, Waters Chromatography B.V, Etten-Leur,
448 The Netherlands; TSP UV6000LP UV-detector (wavelength: 260 nM) Thermo Scientific, The
449 Netherlands). Samples for peak isolation were separated on a Vydac Semi-preparative C18 column
450 (218TP510, 5 µm, 300 Å, 10 mm × 250 mm, VWR International B.V, Amsterdam, The Netherlands) at
451 3 ml/min using the same gradient as above. Data recording and analysis was performed using
452 Chromeleon software (version 6.8 SR13).
453

454 **Catechol extraction from stool for DHPPA quantification**

455 Catechols were extracted from PD patients and HC stool samples using activated alumina powder
456 (199966, Sigma) as previously described (van Kessel et al., 2019) with a few modifications. A volume
457 of 200 µl 50% stool suspension (described above) was used with 1 mM DHBA (3,4-
458 dihydroxybenzylamine hydrobromide, 858781, Sigma) as an internal standard. Samples were adjusted
459 to pH 8.6 with 800 µL TE buffer (2.5% EDTA; 1.5 M Tris/HCl pH 8.6) and 5–10 mg of alumina was
460 added. Suspensions were mixed on a roller shaker at room temperature for 20 min and were sequentially
461 centrifuged for 30 s at 20,000× g and washed three times with 1 mL of H_2O by aspiration. Catechols
462 were eluted using 0.7% $HClO_4$ and filtered before injection into the HPLC-ED-system as described
463 above (DC amperometry at 0.8 V). A standard curve was injected to quantify the concentrations of
464 DHPPA in 50% (w/v) stool samples.
465

466 **LC-MS**

467 HPLC-MS analysis was performed using an Accella1250 HPLC system coupled with the benchtop ESI-
468 MS Orbitrap Exactive (Thermo Fisher Scientific, San Jose, CA, USA) in negative and positive ion mode.
469 Samples were analyzed on a C18 column (Shim Pack Shimadzu XR-ODS 3 × 75 mm) using a gradient
470 of water/acetonitrile with 0.1% formic acid (0-5 min, 98−90% H_2O; 5-10 min, 90-5% H_2O; 10-13 min
471 5% H_2O; 13-14 min 98% H_2O). Data analysis was performed using Qual Browser Thermo Xcalibur
472 software (version 2.2 SP1.48).

473 HPLC-MS analysis of alumina extraction samples was performed using an Waters Acquity Class-I
474 UPLC (Waters Chromatography B.V, Etten-Leur, The Netherlands) system coupled to a MaXis Plus Q-
475 TOF (Bruker, Billerica, MA, USA) on negative ion mode with post-column addition of 3 µl/min ESI
476 Tune Mix (G1969-85000; Agilent Technologies, Middelburg, The Netherlands) for mass calibration.
477 Samples were analyzed on a C18 column (Shim Pack Shimadzu XR-ODS 3 × 75 mm) using a gradient
478 of water/acetonitrile with 0.1% formic acid (0-5 min, 98−90% H_2O; 5-10 min, 90-5% H_2O; 10-13 min
479 5% H_2O; 13-15 min 2% H_2O; 15-17 min 98% H_2O). Data analysis was performed using Bruker Compass
480 Data Analysis (version 4.2 SR1).

481

482 **NMR**

483 Samples were exchanged once with 99.9 atom% D2O with intermediate lyophilization, finally dissolved
484 in 650 µL D_2O. One-dimensional ^1H NMR was recorded at a probe temperature of 25°C on a Varian
485 Inova 500 spectrometer (NMR Department, University of Groningen). ^1H chemical shifts are expressed
486 in ppm in reference to external acetone (δ ^1H 2.225). 1D 500-MHz ^1H NMR spectra were recorded with
487 5000 Hz spectral width at 16 k complex data points, using a WET1D pulse to suppress the HOD signal.
488 Spectra were processed using MestReNova v9.1 (Mestrelabs Research SL, Santiago de Compostela,
489 Spain).

490 **Organ-bath experiments**

491 Distal ileal samples were harvested from wild type adult (18 weeks) male C57BL/6 mice (n=3) that
492 were sacrificed for another purpose. Harvested tissue was immediately removed, placed and washed in
493 0.85% NaCl. Approximately 3 mm rings were cut and were placed in an organ bath (Tissue Bath
494 Station with SSL63L force transducer, Biopac Systems Inc. Varna, Bulgaria) filled with Krebs–
495 Henseleit solution (NaCl, 7.02 g/L; KCl, 0.44 g/L; $CaCl_2.2H_2O$, 0.37 g/L; $MgCl_2.6H_2O$, 0.25 g/L;
496 $NaH_2PO_4.H_2O$ 0.17 g/L; Glucose, 2.06 g/L; $NaHCO_3$, 2.12 g/L) gassed with Carbogen gas mixture (5
497 % CO_2, balance with O_2) at 37 °C. Ileal rings were equilibrated for at least 45-60 min with
498 replacement of Krebs–Henseleit solution approximately every 15 min. Sequentially, 50 µM of
499 acetylcholine (ACh) (Sigma, A2661) was added to induce a stable repetitive muscle twitch response,
500 and after ~5 min 100 µM of DHPPA (Sigma, 102601) was added for ~15 min before the ileal rings
501 were washed. This step was repeated 2-4 times per ileal preparation. As control, ACh was added for at
502 least 20 min with or without 0.05% ethanol (solvent of DHPPA) after 5 min to check for spontaneous
503 decrease. Data was recorded and analyzed in BioPac Student Lab 4.1 (Build: Feb 12, 2015).

504 **Availability of data and materials**

505 All data generated or analyzed during this study are included in this published article and its
506 supplementary information files.

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