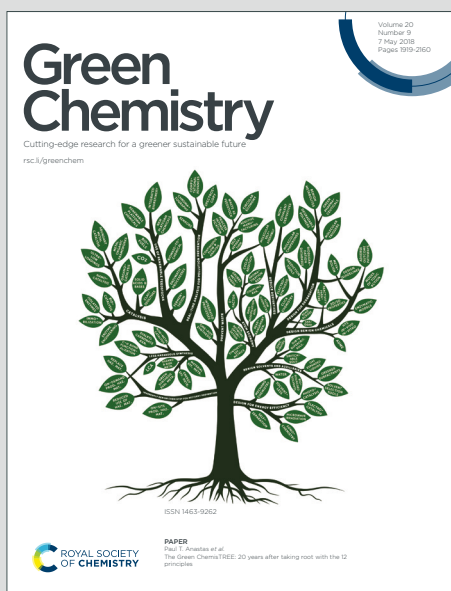


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COMMUNICATION

Development of a *Corynebacterium glutamicum* bio-factory for self-sufficient transaminase reactions.pc36Received 00th January 20xx,
Accepted 00th January 20xxStylios Grigoriou,^a Pierre Kugler,^b Evelina Kulcinskaja,^a Frederik Walter,^a John King,^a Phil Hill,^a Volker F. Wendisch,^b and Elaine O'Reilly^{*a,c}

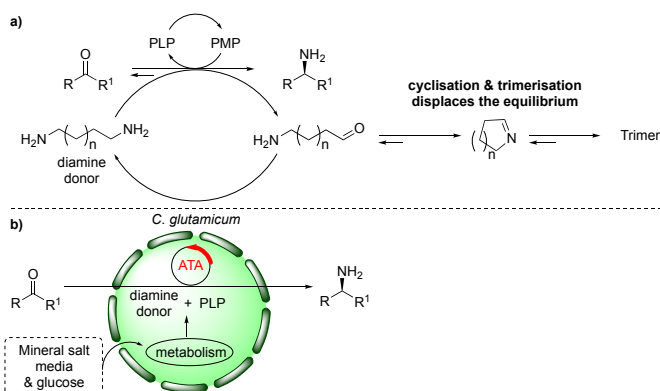
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The development of biocatalytic routes for the synthesis of chiral amines starting from achiral building blocks is highly desirable. Here, we report a self-sufficient whole-cell system for the conversion of a model ketone to the corresponding cyclic imine, in good isolated yield (42%) and excellent enantioselectivity (>99% ee). The *Corynebacterium glutamicum* host produces the transaminase biocatalyst, cofactor and 'smart' amine donor (cadaverine or putrescine) *in vivo*, and highlights the potential for producing high-value chemicals from readily available building blocks. The report represents the first example of the application of a metabolically engineered organism for the production of smart diamine donors and their application in a transaminase biotransformation.

Rapid progress in synthetic biology has enabled the development of efficient biocatalytic systems for the production of fine chemicals and amino acids.¹⁻⁷ The exceptional levels of regio- and stereoselectivity associated with enzymes means that extremely efficient routes to target molecules can be devised that avoid the need for protecting group manipulations and costly intermediate purification steps and enable the design of multi-enzyme cascades.⁸⁻¹¹ The aqueous media and mild conditions employed can provide a cost-effective and sustainable synthetic route to target molecules. As protein purification can be expensive and time-consuming, the application of whole-cell systems represents an attractive alternative to isolated enzymes and significantly reduces upstream costs.^{12,13} The stable intracellular

environment of the host cells, as well as the ability to regenerate essential cofactors and/or co-express multiple enzymes in a single organism, constitute attractive features for synthetic applications.

Amine transaminases (ATAs) represent an extremely important enzyme family for the synthesis of chiral amines. These proteins rely on pyridoxal 5'-phosphate and a sacrificial amine donor to convert a range of aldehydes, ketones and ketoacids to the corresponding (chiral) amines or amino acids.¹⁴⁻²⁰ In recent years, considerable effort by our own group and others has focused on the development of methodology that effectively displaces the challenging reaction equilibrium towards product formation.²¹⁻²⁵ The use of 'smart' amine donors, where coproducts tautomerize,²¹ dimerize,²⁵ cyclize^{22,23} and polymerize,²⁴ have been shown to enable high conversions to the desired amine product. Our group recently reported that cadaverine functioned as an excellent amine donor in transaminase-mediated biotransformations and allowed the conversion of a series of ketones to the corresponding chiral amines (Scheme 1 (a)).^{22, 26} Putrescine has also been shown to act as an amine donor for some ATAs.²³



Scheme 1. a) Use of 'smart' diamine donors for effective equilibrium displacement of transaminase-mediated biotransformations.^{22,23,26} b) Proposed whole-cell

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biotransformation, using engineered *C. glutamicum* cells that produce diamine donors.

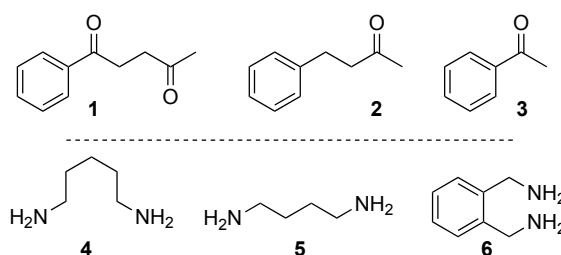
Corynebacterium glutamicum has been used for the industrial production of L-amino acids for decades and more recently been applied for the preparation of a wide variety of small molecules and polymers.^{7, 27-29} *C. glutamicum* ATCC13032 was engineered to produce putrescine (1,4-diaminobutane)^{30,31} (strain named NA6) and cadaverine³² (1,5-diaminopentane) (strain named Cada1), starting from glucose and ammonium salts. We envisaged that these strains could be employed for the design of a whole-cell biocatalyst, capable of the *in-situ* production of the ATA, PLP coenzyme and 'smart' amine donor (Scheme 1(b)). Kim *et al.* previously reported a recombinant *C. glutamicum* strain that was engineered to overproduce non-natural amino acids *via* a transaminase reaction.³³ While the work highlighted the potential of self-sufficient systems, the α -transaminase employed is limited to α -amino and α -keto acid substrates and the report does not include the isolation of final products. The flexible system proposed herein allows the preparative-scale synthesis of high-value chiral products, starting from inexpensive sources, and represents the first example of the production and application of a self-sufficient whole-cell system, overproducing smart amine donors.

In order to identify suitable ATA candidates for the *C. glutamicum* system, a small panel of (*R*)- and (*S*)-selective ATAs were screened against the 'smart' amine donors cadaverine, putrescine and *o*-xylylenediamine (table S1). *O*-xylylenediamine was included for comparison and also to establish if the compound could be used for in a colorimetric assay to identify efficient *C. glutamicum* whole-cell systems. Three ketones (**1-3**) were selected to explore the efficiency of these donors, based on the difficulty associated with displacing the reaction equilibrium towards product formation.^[24] Negligible activity was observed for the (*R*)-selective ATAs with all substrates. Four (*S*)-selective ATAs were identified from *Silicibacter pomeroyi* (referred by its PDB code: 3HMU),³⁴ *Pseudomonas* species namely; *P. chlororaphis* ssp. *aerofaciens* (*PcATA*), *P. fluorescens* (*PfATA*) and *P. putida* (*PpATA*),²³ which were capable of mediating the transamination of ketones **1-3** in poor to high conversion (up to 75%), using only 1 equivalent of amine donor **4-6** (table 1). Significantly, 3HMU achieved conversions of >70% with diketone **1**, regardless of the amine donor employed. The other ω -TAs screened were considerably more efficient at converting **1** to the corresponding amine using cadaverine (40-55%), compared to those conversions achieved when putrescine or *o*-xylylenediamine was employed as the amine donor. Ketone **2** was relatively poorly converted in all cases, with a maximum of 25% conversion achieved when *PfATA* was used in combination with cadaverine. Previous reports employing higher enzymatic loadings with these ATAs have shown comparable conversions of **2** to the corresponding chiral amine in the presence of 'smart' amine donors.^{23,25} To effectively demonstrate the methodology, the transamination of ketone **1** with 3 equivalents of cadaverine utilizing 3HMU, *PcATA*, *PfATA* or *PpATA* was explored (Table 1, entry 10). A

significant improvement was observed, reaching up to 99% conversion with 3HMU and 80% with *PcATA*.

Next, the *in vivo* production of 'smart' amine donors **4** and **5** by *C. glutamicum* was examined, by monitoring the conversion of model ketone **1** to the corresponding cyclic imine, using the bacterial culture medium as the sole source of amine donor. For these early studies, strains expressing the ATA genes were not employed, but rather the enzyme was supplied externally. The biotransformations with *C. glutamicum* strains (GRLys1, NA6 and Cada1) were carried out in conditioned medium (cell-free lysate) of one- or two-day mature culture, supplemented with purified ATA (1 mg mL⁻¹) and ketone **1** (5 mM) (see Table S2). In all cases, conversions were significantly higher (60-98%) than those achieved when 1 equivalent of donor **4** was exogenously supplied (Table 1) and were comparable to those achieved using 3 equivalents of the same donor (Table 1, Entry 10). Interestingly, up to 15% conversion was observed for biotransformations involving the conditioned media from *C. glutamicum* GRLys1. GRLys1, which is used in this study, has been engineered to overproduce L-lysine,² and conversions of **1** to the corresponding imine in the presence of GRLys1 media

Table 1. Initial screen to establish the amine donor (**4-6**) substrate scope of a small panel of TAs, by evaluating their ability to convert ketones **1-3** to the corresponding chiral amine. Clarified crude extract (1.5 mg mL⁻¹) and 1 equiv. 'smart' amine donor. *Ee* values for all were >99%, with the exception of ketone acceptor **2**, where *ee* values were between 80-85% (see Table S1 for full details).



Entry	Ketone	Amine	Eq.	Conversion (%)			
				3HMU	<i>Pc</i> ATA	<i>Pf</i> ATA	<i>Pp</i> ATA
1	1	4	1	75	43	54	53
2	1	5	1	72	20	28	24
3	1	6	1	74	23	35	25
4	2	4	1	21	14	25	18
5	2	5	1	19	6	9	9
6	2	6	1	20	8	17	11
7	3	4	1	nd	nd	9	nd
8	3	5	1	nd	nd	nd	nd
9	3	6	1	nd	nd	7	nd
10	1	4	3	99	80	78	75

Conversions of 5 mM of **1**, **2** or **3** to the corresponding chiral amine utilizing 1.5 mg mL⁻¹ clarified crude extract 3HMU, *Pc*, *Pf* or *Pp* ATA, 0.1 mM PLP and 5 or 15 (entry 10) mM cadaverine at 30°C, 200 rpm, 24h.

likely arise due to L-lysine acting as an amine donor. This amino acid donor itself has the potential to function as a 'smart' amine donor, since the coproduct has the ability to cyclise and displace the reaction equilibrium towards product formation.³⁵ All four ATAs were tested on ketone **1**, utilizing 1 or 3 equivalents of L-lysine as the amine donor (Table S3). However, only 3HMU showed measurable conversion (up to 16%), supporting our observations in the presence of *C. glutamicum* GRLys1.

Subsequently, the four ATA candidates were cloned into the engineered strains with the aim of generating a self-sufficient whole-cell system. A solid-phase screen, employing *o*-xylylenediamine,²⁴ was used to detect transaminase activity from the newly transformed strains (Figure 1). Dark colonies were observed for *Pc*, *Pf* and *Pp*ATA, indicating transaminase activity. However, no colour change was observed for strains transformed with the 3HMU gene. To explore possible reasons for the lack of ATA activity with 3HMU, further investigations on transformants harboring this gene were carried out. Initially, the 3HMU sequence was investigated for the presence of rare codons, but this was quickly ruled out. We hypothesized that potential toxicity associated with 3HMU activity could hamper the expression or lead to inactive mutants. To that end, the catalytic lysine residue (K292) was mutated to alanine, using site-directed mutagenesis. The resulting 3HMU variant was expressed in *E. coli* BL21-(DE3) and its activity assessed using the acetophenone assay³⁷ (data not shown). As expected, the mutant displayed no detectable activity. Gene expression in *C. glutamicum* strains was examined using SDS-PAGE and MALDI-TOF analysis, and the results showed that no detectable quantity of the inactive mutant was expressed (data not shown). At this point, we rationalized that complications at a transcription level may hamper 3HMU expression, but this was not investigated further in this study and instead, our efforts concentrated on the active putrescine ATAs that gave colored colonies.

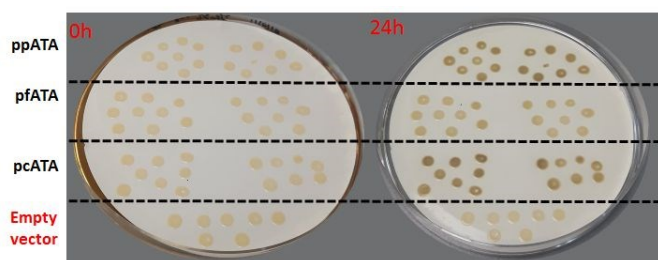


Figure 1. Sample plate showing the emergence of dark colonies when *o*-xylylenediamine is used in combination with recombinant *C. glutamicum* strains.

Positive transformants of both putrescine (NA6) and cadaverine (Cada1)-producing *C. glutamicum* strains, overexpressing the *Pc*, *Pf* or *Pp* ATA genes, were carried forward and tested under biotransformation conditions at both 30 °C and 37 °C (Table S4). However, both strains performed poorly, with a maximum

conversion of 12% observed for *C. glutamicum* NA6 overexpressing the *Pc*ATA (Table S4). We speculated there were two reasons for the observed poor performance of these ATAs. Firstly, the ATAs studied retain less than 20% activity in the intracellular neutral pH. This was verified by measuring the specific activity at different pH values of purified extracts of *Pc*, *Pf* and *Pp* ATAs utilizing the acetophenone assay (Figure S3). Secondly, a low copy number plasmid, pEKEx3,^{38,39} was used to host the ATAs, due to its compatibility with the pre-existing plasmid pVWEx1,⁴⁰ which harbors the genes encoding the lysine decarboxylase (*ldcC*) or ornithine decarboxylase (*speC*) that are essential for the production of cadaverine^[32] and putrescine,^{30,31} respectively. Following previous reports in the literature,⁸ we envisaged enhancing gene expression by introducing a second copy of the *Pc*ATA gene into the pEKEx3 vector. A slight increase was observed, reaching up to 32% after 48 h in the presence of the *C. glutamicum* NA6 strain, which produces putrescine (Table S5). In order to further optimize the whole-cell system, we sought to co-express the ATAs with the *ldcC* or *speC* in the high-copy number vector pVWEx1. Due to the size of the new plasmids, the production of putrescine was compromised and the less efficient NA2b strain was used instead. The NA2b strain has also been engineered to produce putrescine, but it does so less efficiently than the NA6 strain of *C. glutamicum*.

The NA2b and GRLys1 strains, harboring the high copy number plasmid and *ldcC* or *speC* genes were tested under the same reaction conditions. A significant drop to 8% was observed for all the NA2b transformants, whereas conversions significantly increased for the GRLys1 strains, reaching up to 41% (Table S6).

C. glutamicum GRLys1 co-expressing *ldcC* and *Pc*ATA in the pVWEx1 vector was identified as the best whole-cell system, and further optimization was carried out. We envisaged that using a higher concentration of cells would correspond to more biocatalyst and result in increased conversions. The conversion of diketone **1** to pyrroline **7** was evaluated, using various concentrations of cells resuspended in HEPES buffer or in the original culture media. The aim of this study was to establish if the amine donor was being utilized from within the cell or after it has been released into the culture media. Conversions reached a plateau at 73-77%, when 100 mg mL⁻¹ or more of wet cells were employed (Figure 2). The addition of external PLP was also tested, but this had no impact on the conversions, indicating that *C. glutamicum* cells are producing sufficient coenzyme to support the expressed ATAs. In parallel, biotransformations were carried out in recovered culture media, which afforded comparable conversions. These results demonstrate cells either remain metabolically active and continue producing cadaverine, or the intracellular levels of cadaverine are high enough for the biotransformation to occur.

The reusability of this system was tested, by utilizing the cells in three consecutive cycles (Table S7). The cells were washed after each cycle to ensure no product was carried forward and this was verified by GC-FID. The results indicate that the whole cells can be reused once, with minimal loss in activity, whereas a significant drop to 22% was observed during

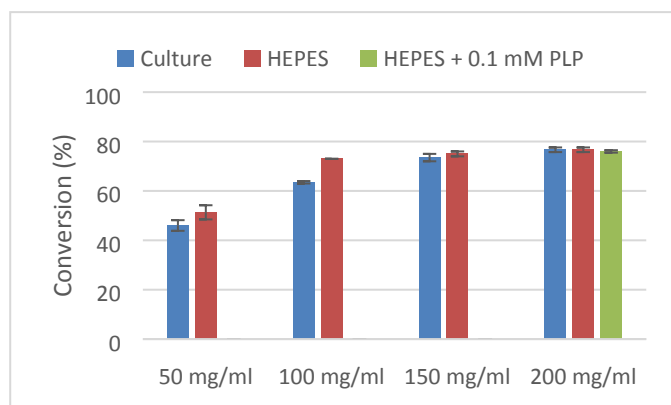
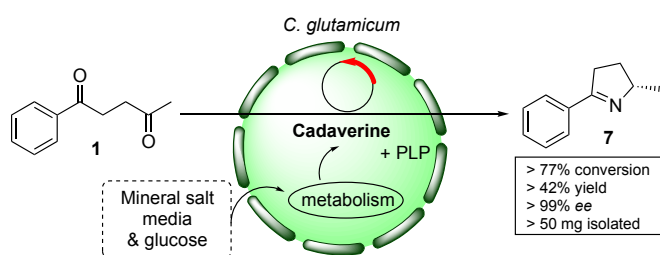


Figure 2. Conversion of **1** in the presence of different cell densities of *C. glutamicum* GRLys1 whole-cells, overexpressing *ldcC* and *PcATA*.



the third cycle. This decrease in activity is unsurprising, and likely to be a result of diminishing enzyme activity as well as a reduction in the levels of amine donor available, in the absence of culture media.

To demonstrate the industrial potential of our system, a preparative-scale biotransformation, utilizing 100 mg mL⁻¹ wet cells in buffer, was performed on the model ketone substrate **1** (5 mM) affording the enantiomerically pure (>99% ee) (S)-pyrroline **7** with 42% isolated yield (Scheme 2).

Scheme 2. Preparative-scale biotransformation of **1** to imine **7**, using 100 mg mL⁻¹ of wet cells.

Conclusions

In summary, we have developed the first whole-cell system for the co-production of an ω-TA and sacrificial ‘smart’ amine donor. Optimization of reaction conditions for the best biocatalyst enabled the preparation of multi-milligram quantities of a chiral pyrroline product, starting from the corresponding commercially available pro-chiral diketone. Our system has the potential to provide a sustainable platform for the development of an array of whole-cell ω-TAs biocatalysts that rely on smart amine donors to displace the challenging reaction equilibrium towards product formation, negating the need for the external supply of co-substrate. Before this goal can be realized, the activity of a wide range of ATAs towards a

more diverse ketone panel must be evaluated in these engineered *C. glutamicum* cells, as high-conversion using this methodology is currently limited to one model substrate. However, we are extremely confident that excellent activity towards other ketones can be achieved with alternative ATAs, based on previous work reported in our group, which shows high conversions with just 1 equivalent of these smart amine donors. Significantly, this work demonstrates that better conversions are achieved using conditioned media containing the diamines, compared to those afforded when 1 equivalent of the amine donor is exogenously supplied (Table S2). The putrescine ATAs (*Pc*, *Pf* and *PpATA*) used in this study require very high enzyme loadings for good activity towards ketones and this goes some way to explaining why substrates **2** and **3** were not efficiently converted to the corresponding chiral amines when expressed in engineered *C. glutamicum* cells. With further development, this system could be used to develop *in vivo* biocatalytic cascades towards high-value chiral molecules, by the introduction of additional genes into the *C. glutamicum* cells.

Our system already performs broadly well (non-hazardous solvents, use of catalysts, health and safety) when assessed using a recently developed Zero Pass Metrics Toolkit, which aims to evaluate the ‘greenness’ of a process.⁴¹ However, it is also important to note that these metrics are not easily applied to *in vivo* systems, where reagents, catalysts and cofactors are produced by the host cell and highlights the need for a set of guidelines to assess the sustainability of whole-cell systems.

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Conflicts of interest

There are no conflicts to declare.

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