Establishment of a novel synthetic biological system based on the periplasmic oxidation system of acetic acid bacteria for the production of toxic aldehydes

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Research objective

The microbial production of valuable compounds has advanced in recent years owing to progresses in synthetic biology. However, reports on the microbial production of toxic compounds, particularly aldehydes, remain extremely limited. Therefore, novel strategies are needed to improve the practical feasibility of achieving the bioproduction of such compounds. In this study, we focused on the periplasmic oxidation system of acetic acid bacteria (Figure 1A). Because this system operates outside the cytoplasm, it is advantageous for targeting compounds that exhibit intracellular cytotoxicity. Moreover, the system consists of dehydrogenases with broad substrate specificity,[1)] rendering it useful for producing non-natural compounds through pathways not found in nature. Accordingly, the aims of this study were to identify membrane-bound dehydrogenases from acetic acid bacteria capable of producing butyraldehyde and the non-natural compound 3-hydroxybutyraldehyde and to demonstrate the fermentative production of these aldehydes using a co-culture system of acetic acid bacteria and *Escherichia coli* (Figure 1B).

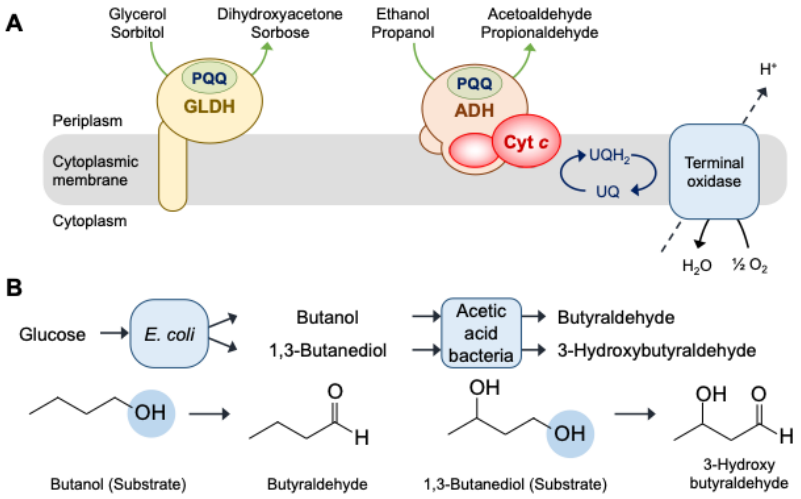

Fig. 1. Periplasmic oxidation system of acetic acid bacteria (A), Proposed co-culture systems of acetic acid bacteria and *E. coli* (B).
Glycerol dehydrogenase (GLDH), Alcohol dehydrogenase (ADH): Representative dehydrogenases.
The circled moiety in the structural formula indicates the functional group oxidized by acetic acid bacteria.

Methods

We used a library of dehydrogenase-deficient *Gluconobacter japonicus* mutants to explore candidate membrane-bound dehydrogenases. This library, which was originally constructed in our laboratory, was generated by sequentially deleting six major membrane-bound dehydrogenases. Membrane fractions were prepared from these mutants, and the dehydrogenase activities toward butanol and 1,3-butanediol were measured using an artificial electron acceptor to identify the target enzymes. To elucidate the reaction products of the identified enzymes, we examined *G. japonicus* and other acetic acid bacteria (viz., *Acetobacter pasteurianus* and *Gluconacetobacter diazotrophicus*) whose genomes are known to encode the target enzymes. Substrate conversion assays were performed using membrane fractions, and both substrates and products were quantified using high-performance liquid chromatography (HPLC).

Additionally, co-cultures were established by inoculating both a butanol-producing *E. coli* strain[2)] and acetic acid bacteria harboring the target dehydrogenases into the same fermentation system. A similar system with 1,3-butanediol producers was planned but could not be implemented within the available timeframe. Bacterial cultivation was performed in jar fermenters under batch conditions with the inoculation ratio of the two strains adjusted. The culture conditions were set to allow the growth of both species, and the substrates and products were quantified using HPLC.

Results

Activity assays using membrane fractions from cells of the mutant library revealed that the oxidation of butanol and 1,3-butanediol was markedly decreased by the deletion of alcohol dehydrogenase (ADH). The additional deletion of glycerol dehydrogenase (GLDH) also caused a substantial decrease in 1,3-butanediol oxidation. These results indicate that ADH plays a primary role in the oxidation of both substrates, and GLDH also contributes to 1,3-butanediol oxidation. Analysis of the reaction products from butanol oxidation revealed that *G. japonicus* did not accumulate the target compound butyraldehyde but instead produced butyrate quantitatively (Figure 2A). This suggests that ADH in *G. japonicus* completely oxidizes butanol to butyrate via butyraldehyde. To compare the enzyme characteristics across species, ADHs from other acetic acid bacteria were tested. *Ga. diazotrophicus* ADH enabled the accumulation of butyraldehyde (Figure 2B), whereas *A. pasteurianus* ADH first led to the transient accumulation of butyraldehyde followed by its decrease and concomitant butyrate formation (Figure 2C). These results indicate that although ADHs are widely conserved among acetic acid bacteria, their biochemical properties vary, and *Ga. diazotrophicus* ADH is particularly suitable for butyraldehyde production. In *G. japonicus*, ADH produced 3-

hydroxybutyraldehyde and 3-hydroxybutyrate when 1,3-butanediol was used as the substrate, whereas GLDH generated 4-hydroxy-2-butanone (Figure 2D, some data not shown). Notably, *G. japonicus* ADH led to a significant accumulation of 3-hydroxybutyraldehyde, indicating that it can be used for the production of this non-natural compound.

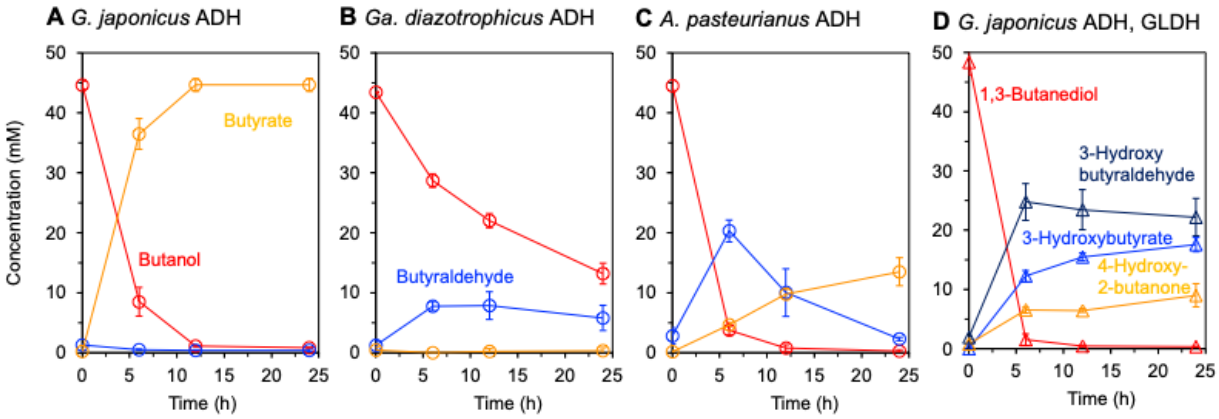

Fig. 2. Butanol (A-C) and 1,3-butanediol (D) conversion assays using membrane fractions of dehydrogenases. Assays with ADHs from *G. japonicus* (A), *Ga. diazotrophicus* (B), and *A. pasteurianus* (C), and ADH and GLDH from *G. japonicus* (D).

Finally, aldehyde production was examined in co-culture systems. A butanol-producing *E. coli* strain and *G. japonicus* expressing *Ga. diazotrophicus* ADH were co-cultured at varying inoculation ratios. Under conditions where both *E. coli* and acetic acid bacteria were inoculated at an initial OD_{600} of 0.1, butyraldehyde production of approximately 0.7 mM was detected after 45 h.

Conclusion

In this study, we aimed to establish a synthetic system for the bioproduction of toxic aldehydes by exploiting the unique features of the periplasmic oxidation system of acetic acid bacteria. We identified dehydrogenases responsible for the production of butyraldehyde and 3-hydroxybutyraldehyde. In particular, ADH from *Ga. diazotrophicus* proved to be effective for butyraldehyde production, whereas ADH from *G. japonicus* was suitable for generating 3-hydroxybutyraldehyde. Furthermore, we demonstrated the fermentative production of butyraldehyde, albeit in small amounts, in a co-culture system comprising *E. coli* and an acetic acid bacterium. Future work will focus on analyzing the growth dynamics in co-cultures, monitoring changes in the membrane-bound dehydrogenase activity of acetic acid bacteria during cultivation, and optimizing culture conditions to improve productivity. Additionally, we will pursue the production of 3-hydroxybutyraldehyde, ultimately aiming to establish a new microbial platform for aldehyde biosynthesis based on the periplasmic oxidation system of acetic acid bacteria.

References

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2) Kataoka, N. et al. (2015) Construction of CoA-dependent 1-butanol synthetic pathway functions under aerobic conditions in *Escherichia coli*. *J. Biotechnol.* **204:** 25–32.