APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1988, p. 1889–1891
0099-2240/88/071889-03$02.00/0

Vol. 54, No. 7

Cloning and Expression of an α-Acetolactate Decarboxylase Gene from *Streptococcus lactis* subsp. *diacetylactis* in *Escherichia coli*

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Received 28 January 1988/Accepted 19 April 1988

The *Streptococcus lactis* gene coding for α-acetolactate decarboxylase (ADC) was cloned in *Escherichia coli*. Subsequent subcloning in *E. coli* showed that the ADC gene was located within a 1.3-kilobase DNA fragment. The ADC gene was controlled by its own promotor. Gas chromatography showed that *S. lactis* and the transformed *E. coli* strains produced the two optical isomers of acetoin in different ratios.

Diacetyl (2,3-butanedione), one of the major compounds causing off-flavor in beer, is formed by a nonenzymatic reaction from α-acetolactate, an intermediate compound of the isoleucine-valine-pathway (Fig. 1). During beer maturation, which may require several weeks, diacetyl is enzymatically reduced by yeast to acetoin and subsequently to 2,3-butandiole, which, in contrast to diacetyl, does not influence beer flavor.

The direct conversion of α-acetolactate into acetoin is catalyzed by α-acetolactate decarboxylase (ADC). This enzyme is found in various procaryotes, for example, *Streptococcus lactis* subsp. *diacetylactis* (6) and *Enterobacter aerogenes* (13, 16) but is not present in yeasts or other eucaryotes (9). Addition of ADC to the fermenting wort or to freshly brewed beer (young beer) can dramatically reduce the formation of diacetyl, allowing a shorter lagering time (7, 8, 10).

An alternative method for using ADC in beer maturation could be the utilization of a brewing yeast genetically altered to produce ADC. In order to comply with current food legislation, the donor of the ADC gene must be absolutely harmless and suitable for food production. In contrast to *Enterobacter* spp., which are putrefying bacteria, *S. lactis* is already used in the cheese-making industry. In addition, ADC from *S. lactis* has proved to be much more active in reducing the formation of diacetyl than the *Enterobacter* enzyme when added to fermenting wort (5).

The initial stage in producing such a yeast was to clone the ADC gene of *S. lactis* and demonstrate its expression in *Escherichia coli*. Total cell DNA of *S. lactis* subsp. *diacetylactis* DSM 20384 (11) was partially digested with *Sau3A*, ligated into the *Bam*HI site of pBR322, and transferred to *E. coli* SF 8 (for a description of the host strain, see reference 17). All cloning steps were carried out as described previously (4, 14). Plasmid-carrying *E. coli* clones were selected with ampicillin.

As shown by the Voges-Proskauer (VP) reaction (12), *E. coli* cannot form acetoin or diacetyl when grown on pyruvate as the sole carbon source (Table 1). Among more than 600 *E. coli* transformants carrying pieces of the *S. lactis* genome, 1 was found that gave a positive VP reaction. As this clone, SF8(pADC1), seemed to carry the ADC gene of *S. lactis*, its extrachromosomal DNA was further characterized and its ability to form acetoin was verified by gas chromatography.

In order to demonstrate that the cloned DNA fragment originated from *S. lactis*, hybridization experiments were carried out (14). DNA from *S. lactis* was restricted with *Sau*3A and then hybridized with pADC1 and pBR322. The vector pADC1 hybridized to a 4.5-kilobase (kb) fragment, whereas pBR322 showed no homology to the DNA of *S. lactis* (Fig. 2).

pADC1 carries a 4.5-kb DNA fragment of *S. lactis*, enabling *E. coli* to produce the same amount of acetoin (16 μg/ml) as the source strain (Table 1). By subsequent deletions characterized by restriction analysis, this fragment could be shortened to about 1.3 kb (isolate p31-H1) without affecting the quantity of acetoin produced. Deletions which included this whole area (like pADC4) or part thereof (pADC3 and pADC5) led to a total loss of acetoin production, whereas deletions beyond this area had no effect (pADC2 and p31-3S). It can therefore be concluded that the whole ADC gene must be located on this subfragment. Furthermore, as the orientation of this subfragment relative to the adjacent vector DNA did not influence acetoin formation, it seems plausible to conclude that the ADC gene is under the control of its own promotor.

As described above, to screen for acetoin-forming transformants the VP reaction was used. Since the VP reaction is positive not only with acetoin but also with diacetyl (19) (Fig. 1), it was necessary to confirm whether the positive VP reaction of the transformants was in fact caused by the presence of acetoin. For its identification, the vicinal diketones were extracted with diethyl ether from the culture filtrate of SF8(pADC2), dried with sodium sulfate, concentrated, and separated by gas chromatography (Carbowax 20 M, 70°C/4°C). According to the chromatography data (not shown), only acetoin was present; no traces of diacetyl were found.

To clarify whether the formation of acetoin was in fact catalyzed directly by ADC or indirectly by diacetyl reductase (Fig. 1), the ability of the transformants to convert diacetyl (added instead of pyruvate to the substrate) to acetoin was examined. In all cases, acetoin could not be detected. In addition, other workers (2, 3, 18) have shown that procaryotes, in contrast to eucaryotes, do not contain the pyruvate dehydrogenase complex, which can combine acetaldehyde and pyruvate or two molecules of acetaldehyde (head to head) to form acetoin. It is therefore obvious that the cloned fragment of *S. lactis* must contain the ADC gene. From investigations with *Bacillus polymyxa*, it is known that the proportion of the two optical isomers of acetoin [(*R*)- and (*S*)-acetoin] formed by ADC is strain specific (18). It is not known whether this specificity is solely

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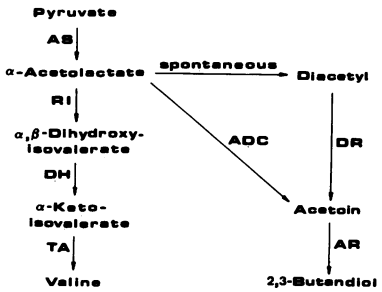

FIG. 1. Main steps of the valine-biosynthetic pathway (1, 15). Dependent on the organism, acetoin may be formed directly from α-acetolactate or via diacetyl which arises spontaneously. Abbreviations for the enzymes catalyzing the individual steps: AS, acetolactate synthase; RI, reductoisomerase; DH, dehydrase; TA, transaminase; DR, diacetyl reductase; AR, acetoin reductase.

dependent on the ADC gene or can also be influenced by the host strain. To examine these two alternatives, the amount of (*R*)- and (*S*)-acetoin was determined in culture filtrates of *S. lactis* and *E. coli* carrying pADC2 by gas chromatography under the following conditions: acetoin was extracted with diethyl ether, derivated with (*R*)-(+)-α-methoxy-α-trifluoromethyl-phenylacetic acid-chloride and separated on a column (DB210, 140°C isotherm). The data obtained showed that *S. lactis* produced about 91% (*R*)- and 9% (*S*)-acetoin (which is in good agreement with the values found in *B. polymyxa*), whereas surprisingly, the transformed *E. coli* strain showed a proportion of 52 and 48, respectively. As a chemical racemerization can be excluded (the culture medium was buffered at pH 7.0), one can suppose either that the ADC expressed by *E. coli* had lost its original stereospecificity (and eventually, by posttranslational modification, became different in the two organisms) or that the host strain contains a racemase which is by chance specific for acetoin. At present it is not possible to determine which of these alternatives is correct.

After having cloned and identified the ADC gene from *S. lactis*, the next step towards a diacetylless brewing yeast will be expression of the ADC gene in *Saccharomyces cerevisiae*, although as yet, no naturally occurring ADC activity is known in eucaryotes. Subsequent fermentation tests will show whether the maturation time of young beer can be reduced by using a genetically engineered yeast strain in the same way as adding the appropriate enzyme (ADC) to fermenting wort.

TABLE 1. Acetoin production by *E. coli* transformants containing pADC1 or various subfragments of pADC1 by shotgun cloning from *S. lactis* genomic DNA[a]

Strains & Clones	Characterization of the Streptococcal DNA Cloned in E.coli	Acetoin (ppm)
S.lactis DSM 20384	Source Strain	16
E.coli SF8	Cloning Strain	not detectable
SF8/pADC1	S P H EP R S 4.5kb	16
SF8/pADC2		16
SF8/p31-38		16
SF8/p31-H1		16
SF8/pADC3		not detectable
SF8/pADC4		not detectable
SF8/pADC5		not detectable

[a] For comparison, the acetoin data for the cloning and source strains are also given. Acetoin was determined spectrophotometrically (at 540 nm) by the VP reaction in culture filtrates (culturing time, 24 h). The limit of detection was 3 ppm of acetoin. The bars in the middle column indicate the cloned streptococcal DNA areas out of the 4.5-kb large DNA fragment shown which was gained from the streptococcal genomic library. Some restriction sites are given: S, *Sau*3A; P, *Pvu*II; H, *Hin*dIII; E, *Eco*RV; R, *Rsa*I.

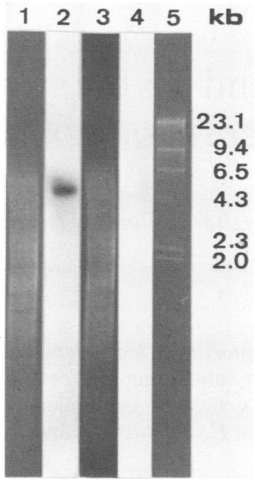

FIG. 2. Hybridization of *Sau*3A-digested *S. lactis* DNA with pADC1 and pBR322. Lanes 1 and 3, *S. lactis* DNA digested with *Sau*3A; lane 2, *S. lactis* DNA hybridized with pADC1; lane 4, *S. lactis* DNA hybridized with pBR322; lane 5, λ DNA digested with *Hin*dIII as size markers.

We thank J. Heidlas for the gas chromatography. We are also indebted to K. Lehmann for technical assistance and to E. Quaite for critical reading of the manuscript.

This research was supported by the Arbeitsgemeinschaft Industrieller Forschung.

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