Expression of the *Zymomonas mobilis* Alcohol Dehydrogenase II (adhB) and Pyruvate Decarboxylase (pdc) Genes in *Bacillus*

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**Abstract.** The genes encoding *Zymomonas mobilis* pyruvate decarboxylase (pdc) and alcohol dehydrogenase II (adhB) were expressed in *Bacillus subtilis* YB886(pLOI500) under the control of a *Bacillus* SPO2 phage promoter and caused a 50% reduction of growth rate compared with the unmodified vector. Expression was further confirmed by Western blots, activity stains of native gels, and in vitro measurements of alcohol dehydrogenase activity. Additional strains of *Bacillus* were also transformed, and all produced similar but low levels of these enzymes. Although higher specific activities will be required for efficient ethanol production, no fundamental barriers exist to the expression of these *Z. mobilis* genes in *Bacillus*.

Two abundant new proteins (ca. mass 33,000 daltons and 14,000 daltons) were observed in Coomassie Blue-stained gels; they are similar in size to the proteins induced by recombinant products in *Escherichia coli*.

The genes encoding pyruvate decarboxylase (pdc) and alcohol dehydrogenase II (adhB) from *Zymomonas mobilis* have been expressed at high levels in several Gram-negative bacteria, effectively redirecting fermentative metabolism to produce ethanol as the primary product [5, 12, 21]. Unlike conventional yeasts and *Z. mobilis*, these engineered bacteria have an extremely broad substrate range and can readily metabolize all pentose and hexose sugar constituents of lignocellulose.

Many Gram-positive bacteria such as members of the genus *Bacillus* [7, 9, 19] contain glycohydrolases, which could prove advantageous for the production of fuel ethanol. Additional advantages of Gram-positive organisms include extensive use by the food industry and GRAS (generally regarded as safe) status. In this study, we have demonstrated that the *Z. mobilis* genes encoding alcohol dehydrogenase (ADHII) and pyruvate decarboxylase (PDC) can be expressed as active enzymes in *B. subtilis* and *B. polymyxa*.

**Materials and Methods**

**Bacterial strains, plasmids, and growth conditions.** The following *B. subtilis* strains were used: YB886 [22], 168 ISP [13], 3636ISP [16], 168 S-87 [Hageman, personal communication], NRC9057 [4], and NRC990 [4]. Other bacterial strains used in this study include *E. coli* DH5α [Bethesda Research Laboratories], and *B. polymyxa* NRC2882 [4]. Plasmids pLOI292 and pLOI295 [12] and pPL708 [10] have been previously described. Cultures were routinely grown at 37°C in Luria broth [15] supplemented with 50 g/L glucose or on Luria agar (15 g/L agar and 20 g/L glucose). Recombinants of *B. subtilis* and *B. polymyxa* were selected on Luria agar containing kanamycin (10 mg/L) and screened on aldehyde indicator plates for the expression of alcohol dehydrogenase activity [12]. Growth of all organisms was monitored at 550 nm with a Spectronic 70 spectrophotometer (Bausch & Lomb, Inc., Rochester, New York).

**DNA manipulations.** Standard methods were used for the purification of plasmid DNA, plasmid construction, and transformation [11, 17]. *Bacillus subtilis* NRC9057 was transformed by electroporation [6]. Digestions with restriction enzymes were carried out as recommended by the manufacturers.

**Gel electrophoresis and immunoblots.** Soluble protein extracts were prepared as described previously [3] from cultures grown for 8 h. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) essentially as described by Laemmli [14] and either stained with Coomassie Brilliant Blue or electroblotted to nitrocellulose membranes for Western analysis [1, 3]. Zymograms of native polyacrylamide gels (8% acrylamide) were stained for ADHII [20] and PDC [23] activities.

**Enzyme assays.** The specific activities of PDC and ADHII were determined in cell-free extracts as previously described [12].
Fig. 1. Bacillus vector pLOI1500 for the expression of the Z. mobilis pdc and adhB genes (pet operon). The Z. mobilis genes are expressed under the control of a promoter (P) from phage SPO2.

Table 1. PDC and ADHII activities in recombinant strains of E. coli DH5α and B. subtilis YB886

<table>
<thead>
<tr>
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<th>Specific activity&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
<td></td>
<td>ADHII</td>
</tr>
<tr>
<td>E. coli DH5α</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>DH5α(pLOI292)</td>
<td>0.81</td>
</tr>
<tr>
<td>DH5α(pLOI1528)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.6</td>
</tr>
<tr>
<td>B. subtilis YB886</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>YB886(pLOI1500)</td>
<td>0.17</td>
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* Expressed as μmoles of substrate/min per mg of protein [12].
* 3.2 kbp containing the pet operon subcloned from pLOI1500 into pUC18.
* Not determined.

Results and Discussion

Plasmid construction and transformation. A promoterless pet operon was isolated as a 3.2-kilobase pair (kbp) BamHI fragment from pLOI292. This fragment was ligated into the BamHI site of the Bacillus expression vector, pPL708, under the control of the spo promoter [18] to produce pLOI1500 (Fig. 1). To confirm that the Z. mobilis genes were not altered during construction or maintenance in B. subtilis YB886, the 3.2-kbp BamHI fragment was resolated from YB886(pLOI1500) and subcloned into pUC18 to produce pLOI1528. PDC and ADHII activities in E. coli DH5α (pLOI1528) (Table 1) were equivalent to those expressed by an analogous construct, pLOI295 [12], the source of the pet operon for pLOI292.

Expression of proteins encoded by Z. mobilis genes. YB886(pLOI1500) grew at approximately half the rate of the parent organism containing the unmodified vector. The expression of both Z. mobilis pdc and adhB was confirmed immunologically in colony lifts with polyclonal antisera [1]. No new proteins that corresponded in mass to Z. mobilis PDC (59,998 daltons) or ADHII (40,141 daltons) were observed in Coomassie Blue-stained SDS–PAGE gels of YB886(pLOI1500) (Fig. 2A). However, Western blots (Fig. 2B and 2C) revealed the presence of full-length subunits for both PDC and ADHII. Two new smaller proteins were observed in stained gels, ca. mass of 14,000 (14kDa) and 33,000 (33kDa) daltons. It is unlikely that these smaller proteins are degradation products of Z. mobilis enzymes, because both failed to react with either polyclonal antibody. The 14kDa and 33kDa proteins were present only in YB886 recombinants that expressed the Z. mobilis genes. Deletion of the spo promoter (EcoRI fragment) to produce pLOI1503 eliminated their expression, the inhibition of growth, and the expression of the Z. mobilis genes in recombinant YB886.

A second, higher-molecular-weight band was also detected in YB886(pLOI1500) with antisera to ADHII, an abundant Z. mobilis stress protein [3]. This band was observed previously in recombinant E. coli harboring only Z. mobilis adhB and appears to represent an incompletely denatured dimeric form [1].

Expression of functional PDC and ADHII. ADHII activity was readily measured in protein extracts from YB886(pLOI1500) but was lower than observed in E. coli DH5α(pLOI292) (Table 1). PDC activity could not be determined in B. subtilis because of the high background levels of native, heat-stable lactate dehydrogenase [8]. The expression of Z. mobilis adhB and pdc as functional enzymes in YB886(pLOI1500) was confirmed, however, by activity stains of native gels (Fig. 3A and 3B, respectively).

Additional plasmids were constructed in an attempt to increase the expression of the Z. mobilis genes in YB886. The promoterless plasmid, pLOI1503, was used as a recipient for 1- to 3-kbp PstI fragments of YB886 chromosomal DNA as a source of native promoters. Although many positive clones were identified in colony lifts, none appeared more active than pLOI1500 (data not shown).